

nature

June 5, 1975

As the Prince of Wales, so the Non-Proliferation Treaty

Across the wires the electric message came:
'He is no better, he is much the same.'

*On the Illness of the Prince of
Wales, afterwards Edward VII*

Attributed to Alfred Austin (1835–1913)

THE Geneva conference to review the working of the Non-Proliferation Treaty ended last weekend after four frustrating weeks in which relatively little apparent success was evident. Hopes that at last the pressure of a deadline might bring the ninety-odd nations to agreement on something substantial came to nothing. Nor even did there seem to be enough in prospect for the conference to adopt the recent European expedient of stopping the clock to allow more time for discussion. Not for the first time has it been noticeable that the only thing more sacred than an international nuclear treaty is an international airline reservation!

If there is disappointment that the review conference did little to make the treaty look more attractive to the nations still outside its orbit (and it must be said that none of the potentially nuclear non-signatories is likely to be lured into the fold in the foreseeable future), this disappointment must be tempered with an element of relief that the participants did manage to stick together and that there were no defections from the treaty.

It was expected that the nuclear powers, particularly the superpowers, would have an uncomfortable month, and much of the first week or so was taken up with trying to spot who was going to emerge as the standard-bearer of the non-aligned and scourge of the nuclear powers. Eventually it was Mexico which assumed this role and took the strongest line, but the hands of the non-aligned were tied by the failure of many of the signatories among the developing countries to appear at the conference. Thus the two-thirds majority which can so often be mustered in United Nations circles against the developed countries was not easily available. Even so, Mexico's mark was firmly left on such issues as the continuing need to negotiate a comprehensive test ban and the possibility of declarations on non-first-use and non-use of nuclear weapons against non-nuclear powers.

The activities of Mrs Thorsonn, the Swedish president of the meeting, came in for much praise. She delayed her intervention with a draft document until half way through

the last week when there was growing despair that anything would emerge at all, particularly from Committee I, charged with reviewing articles devoted to security assurances, measures for disarmament and nuclear-free zones, yet bogged down on procedural matters. Her draft found wide acceptance and eventually led to a consensus statement accompanied, not surprisingly, by a number of reservations from individual nations.

The nuclear powers could have good cause to feel pleased that they had emerged relatively unscathed from their time in the dock (they did have the advantage, one observer noted, of acting simultaneously as judge and jury).

Not so pleased were one or two other countries. Switzerland, Yugoslavia and Romania all made noises about being surrounded by countries armed with tactical nuclear weapons. With good cause did they fear that in time of crisis they might be trampled on without the superpowers caring too much. The most likely to take this complaint further is Yugoslavia and judging by the performance of the last week or so, the possibility of Yugoslavia's withdrawal from the treaty has seriously to be reckoned with. The pioneering work of India in finding a justification for nuclear development through the peaceful explosions route could handily be used by several nations in danger of being overrun.

There are, nevertheless, several positive things to have emerged from Geneva. The cause of international control both of nuclear fuel reprocessing centres and of peaceful nuclear explosion facilities has advanced considerably as a result of the conference. The concept of nuclear-free zones has been extensively aired, even if for the present only Africa seems likely to be a possible candidate for joining Latin America in this category. And there is to be a further review conference in 1980, preceded by an examination in two years' time at the General Assembly of the United Nations of progress in fulfilling the objectives of the treaty.

If this sounds like modesty in the extreme in advancing the cause of arms control, it is, and many will go away fearing that the treaty will only continue to hold together as long as it places no serious demands on its signatories. Perhaps the treaty is too big in its sweep to be able to survive indefinitely. The real paths of progress in the next few years may be back in more detailed fields—regional treaties, restraints on testing and controls on the transfer of material. □



European space comes of age

The European Space Convention has now been formally signed, and the senior staff of the new European Space Agency appointed. Angela Croome reports on how things are now likely to shape up.

SPACE collaboration was one of the first activities to bring together the countries of western Europe in a common enterprise which needed to reach a critical size that none of the partners could afford on their own. That was in the late 1950s, a long time ago. In the event, two organisations were set up, separating ends and means — the European Launcher Development Organisation (ELDO) and the European Space Research Organisation (ESRO); this was a bad beginning and a source of subsequent confusion and discord. There were always critics who urged that launching and experimentation should be kept together and that there should be a single organisation on the lines of NASA. And the mismatch in structure between the two organisations added strength to this argument. Eventually, the progressive escalation of cost and the regression in the practical value of the launcher element, together with rumblings over resignation from ELDO's initiating member, the UK, prompted the setting up in 1966 of a political council straddling both bodies (the European Space Conference (ESC) of ministers) and moves towards rationalisation and amalgamation. That was in 1966.

Rationalisation and amalgamation did not come until April 16, 1975 when the member countries gave political approval to the outstanding points of the European Space Convention, agreed in principle over a year before and formally signed at a meeting of plenipotentiaries in Paris last week. The delay of a year was caused both by having to wait for the outcome of France's review of her scientific spending and because of a lack of unanimity over the appointment of the new agency's director-general, which was linked to the disposal of other senior agency posts. Although the delay has not really mattered much in the long run the senior staff of ESRO, the caretaker body, have been grossly overworked and Europe's space plans have been made to look rather silly yet again. But ESRO can readily wear such nuisances. It has grown immeasurably in stature since the troubles of the mid-1960s, having pulled off several complex satellite projects and become



Gibson

an equal partner with the USA in a major applications project, the aeronautical satellite programme; it has also produced a convincing case for a key element in the forthcoming Space-lab-shuttle operation.

The new budget was adopted and a revised programme put in hand last year in spite of the lack of formal agreement on the convention and the appointment of the director-general. The basic difference between the old ESRO arrangement and the new European Space Agency (ESA) is sharply reflected in the budget; the agency's is to be six times as large as ESRO's was. European collaboration in space became almost overnight "a very different animal" in the words of ESRO's then acting director-general and head of administration, Roy Gibson. In 1973 the budget was 40 million Accounting Units, each unit being roughly equivalent to \$1 and in 1974 it was 240 MAU — so that from last summer (when ELDO had in reality already ceased to exist) ESRO, as ESA caretaker, was disposing of \$1 million every working day. The justification for these increased sums was the adoption of a substantial applications satellite programme. This has been the central plank of the new agency, and by relying for legality on the existing fully ratified ESRO convention it could get going ahead of the birth of the agency itself. It is expected that ratification of the ESA Convention by the 10 member states will take at least two years, during which time ESRO's convention will continue to be the legal base of the agency's activity under a 'gentleman's agreement'.

Roy Gibson was unanimously adopted as director-general by the ESRO Council in March, as were five new directors who now fill posts that

had lain empty for the past year. Inevitably the atmosphere and performance of the new agency will owe much to its first director-general.

What sort of man is Roy Gibson and how does his administrative style compare with that of his two predecessors at ESRO? The first ESRO Director-General, Professor (now Sir) Hermann Bondi, who is at present Chief Scientific Adviser to the Ministry of Defence, may best be described as the ESRO magician. He inherited major crises—and proved a stunning negotiator by simply sweeping recalcitrant ministers, hesitant officials and critical journalists off their feet by his persuasive tongue and instant arguments. He foresaw that ESRO's success would depend on applications satellites and he promoted them tirelessly (especially his particular baby, the aeronautical satellite project), thereby largely overturning ESRO's original remit, which rather puritanically restricted it to scientific research excluding the engineering aspects. He left ESRO healthy, even muscular, but a bit out of breath. The three years under the German scientific civil servant, Dr A. Hocker, was a complete contrast and perhaps gave the organisation the spell of quiet consolidation and development it needed after the Bondi whirlwind. Dr Hocker was so retiring that it was sometimes asked whether ESRO actually had a director-general. On the other hand he was scrupulously impartial and conscientious in the best civil service tradition. No national delegates were 'bamboozled', no 'deals' were made under the Hocker regime.

Roy Gibson has spent his working life (he is 50) in a variety of international administrative posts. He is no civil servant; he more resembles senior management in a multinational company. He is a glutton for work and long hours, and thrives on both. Pre-breakfast briefings and constant commuting by air are a regular pattern of his diary. He explains that the key to such a life is being able to drop off to sleep at any time—a talent he learned as a cipher officer in the Army during the Second World War. Another asset is his proficiency in several languages. As a Colonial officer in Malaya after the war he spoke Tamil; he subsequently picked up his French during 10 years of work on international safeguards for atomic energy with the UK Atomic Energy Authority and he speaks German at home with his half-Austrian half-Swedish wife.

What difference will the formal existence of the ESA make? Gibson puts

first the fact that the agency is charged with rationalising the aerospace facilities of Europe and in particular "improving the world wide competitiveness of European industry"—in the words of the convention. An Industrial Policy Committee (IPC), which will take a long, hard look at the aerospace structure and performance of each member country, is already being formed. "It may need to tell some firms to drop out"—in the transponder/repeater field, for example—should there prove to be a glut of manufacturers. The IPC will hold its first meeting this summer and by the end of the year there should be some answers.

The other aspect of rationalisation is the pooling of national space projects. Gibson is much encouraged by the change in attitude that was apparent at the meeting of European space ministers in April. Everybody expressed themselves keen to "play the card of Europe", in the French minister's words. Previously, member countries upheld national and European interests at the same time. "The ESA objectives cannot be brought off without sacrifice", says Gibson, "and up till now 'sacrifice' is something someone else makes." An early test may be whether the Germans are willing to pool their direct-television broadcast satellite project.

Gibson is not a man for personal publicity though he seems to recognise the need for such an organisation as the ESA to have a face. He is rather clear on the management style that he intends to promote. He envisages a sharpening and streamlining of management responsibility, what he calls a much more integrated management approach. This can come about now that the full directorate (of nine) has been formed—five appointments had to await the decision on the director-general because of the distribution of jobs among member states. "People think it has all been cooked", says Gibson. "It has—but not in the sense that member countries have off-loaded their duds on us. Today we have no 'diplomatic passengers'". His integrated management philosophy he describes as "simplicity born of desperation". He practises a "wide discussion" of any problem among the directors and all other staff involved—an entirely free debate where everyone chips in but once the executive decision is taken he requires complete loyalty of execution. "I expect whole-hearted acceptance even if 49% didn't want that direction beforehand." And if not, then they can go and peddle their matches somewhere else. He admits he feels "very strongly" about a "team approach" but not in terms of vagueness—the term team is often used to shift responsibility on to someone else.

"We simply cannot afford to be transformed into a woolly civil service-type outfit where people are carefully housed [for example] when they are not making a contribution."

How is any tendency of this kind to be prevented? To start with he considers work style of the ESRO-ESA type of operation is an asset. "In contrast to other organisations, for instance the OECD, there is a direct result—hardware." He explains that there is little paperwork involved and a rigid timetable. If things slip in preparing a space project everything goes awry. Consequently people at ESRO work very hard; they are enthusiasts and think nothing of working late into the night. "Most of our people are sold on space and the level of professional scrounging is extremely small." The way to keep things this way is above all to be extremely careful over recruiting. Gibson comments, however, that it is now eight years since people were recommended by member governments "to get them out of the way". The present standard of new staff is very high. The other aspect is tenure: "We need to steer a mid-course between security and bureaucracy". He sees the long-term contract as the key, and although the appointment of ESRO's director-generals was for three years, one senses that Gibson may be set for something more like a decade.

The stature of Europe-in-space is something that Gibson takes very seriously. He feels that the outside world does not appreciate Europe's capability. "No one doubts that Hughes, for instance, can do the whole thing" but it does not occur to them that western Europe as an entity can.

The opportunity offered by the coincidence of the biennial Paris Air Show and the signing of the ESA Convention has not been missed, for example, and a three-day conference and demonstration of European capabilities has been put on for the benefit of key visitors, including some ministers. Non-ESRO projects such as Britain's defence communications link Skynet and the Franco-German regional satellite Symphonie as well as ground-stations were on view to show that Europe has the capability today to "do the whole thing". Part of this preoccupation is concerned with Spacelab, which Europe is building for the American Shuttle programme for the 1980s and to which it will contribute half the first scientific payload. Gibson feels there is a danger of making propaganda over Europe's first entrance into manned spaceflight, as almost equal partners with the USA. Proper use of the possibilities Spacelab offers is much more important and he is not convinced that what this means is any better understood in the USA than in Europe. Spacelab experiments will cost less and the transfer from drawing board to flight fulfilment will be much quicker than the present seven years. They need not be miniaturised nor automatic. Integrating this highly complex programme is a daunting problem which Gibson has spent a lot of time thinking over. Financial, institutional and payload use, as well as development, all require to be effectively tried together.

The success of Spacelab would undoubtedly be the ultimate justification of the new agency — and the climax of Roy Gibson's career as director-general. □

ESA looks ahead

In a few weeks the first satellite under the European Space Agency's new name will be launched.

This year's satellite, COS-B, will carry a single large experiment, capable of detecting gamma rays with energies exceeding 20 MeV. The next scientific satellite planned for launch (in 1976) is GEOS, which will continue the theme of several European satellites in carrying out integrated studies of the magnetosphere. The earlier HEOS II satellite was the first to measure the magnetospheric properties of the polar cusp region, and also discovered the plasma mantle; HEOS I (now in its seventh year) continues to provide information about related phenomena.

But it is the other satellites in ESA's present programme which really point the way ahead for science in space. This path is very

much one of collaboration. On the International Ultraviolet Explorer satellite, also due for launch in 1976, ESA will be collaborating with both NASA and the United Kingdom (so the UK, as a member of ESA will be collaborating with itself!). This satellite should be accessible to any astronomer in much the same way that telescopes on Earth are used by many different members of the astronomical community.

More collaboration with NASA is involved in the International Sun Earth Explorer (ISEE) project, planned for 1977, which also follows the European tradition of investigating the interplanetary medium and Sun-Earth links. And ESA's biggest project, the Spacelab, ties the future of European space research even more closely to that of NASA; so the last satellite in the present programme, EXOSAT, is likely to be ESA's last independent scientific fling, for a good few years at least.

international news

SOME leaders of the scientific community in the United States are happy, for a change, because President Ford has said that he will welcome them back into the inner corridors of power after more than two years of exile. Last week, Ford told a delegation of key Senators and Congressmen that he wants Congress to pass a bill to establish a small science policy office in the White House, headed by a Science Adviser to the President. The details remain to be worked out, however, and it will be several months before such an arrangement is in place.

Ford's decision follows months of agitation by many scientific organisations and by several Members of Congress, who were considerably irked when former President Nixon announced, in January 1973, that he no longer required the services of a full-time science adviser. Nixon dismantled the science advisory apparatus set up by Presidents Eisenhower and Kennedy, conferred the title of Science Adviser on the director of the National Science Foundation—a small agency out in the bureaucratic hinterlands—and severed the main formal link between the scientific community and the White House by scrapping the President's Science Advisory Committee (PSAC).

Those actions drew heavy criticism, chiefly on the grounds that the National Science Foundation is too far removed from the mainstream of government decision-making to play an effective role in shaping and coordinating policies. Another complaint was that Dr H. Guyford Stever, the Director of NSF, was forced to wear two hats, one as science adviser and the other as director of a research agency—a situation which could raise problems of a conflict of interests and which relegated science advice to a part-time activity.

In the face of such criticisms—which have come from no less a body than the National Academy of Sciences, and which also prompted the Senate to pass a bill last year to set up a three-member Council of Science and Technology Advisers in the White House—it is no surprise that Ford has agreed that his predecessor's actions should be reversed. But the nature of Ford's decision and the manner in which it was announced are a bit curious.

In effect, Ford has outlined roughly what he wants, and he has invited Congress to fill in the details and establish the science policy apparatus by legisla-

Science to return to the White House

from Colin Norman, Washington

tion. According to James Cannon, Assistant to the President for Domestic Affairs, Ford "has opted for a single director (of a White House science policy office), someone with consider-

able ability and scientific standing. He will have . . . perhaps a staff of 10 to 15 assistants". Cannon added that the office would probably be equipped with a budget of between \$1 and \$1.5 million a year.

But, aside from acknowledging the fact that science policy will eventually return to the White House, the announcement leaves a host of unanswered questions. According to one source who attended the Congressional briefing, for example, no decision has yet been made on whether the science adviser's interests should include military matters; it is also unclear how the office would interact with other key policymaking bodies in the White House—especially the Domestic Council and the Office of Management and Budget—and left in the air is the question of what will become of the extensive science advisory structure that now exists in the National Science Foundation as a result of Nixon's 1973 re-organisation.

But perhaps the most curious aspect of the announcement is why Ford chose to ask Congress to establish the office by legislation. He could easily have created whatever apparatus he wants simply by issuing an executive order, just as President Kennedy did when he established the Office of Science and Technology in the White House in 1962 (and as President Nixon did when he scrapped it eleven years later). It could be, as one insider suggested, that it is "just Ford's style of government"—he simply wants to bring Congress in on the matter.

A less charitable interpretation, however, is that the Administration considers the matter to be of low priority, and Ford simply wanted to get it off his back. That interpretation is supported by the fact that Ford had asked Vice-President Rockefeller in December last year to make a study of arrangements for bringing science advice into Presidential decision-making but Rockefeller's report had been gathering dust for some time.

The decision to go to Congress on the matter has several important practical implications. To begin with, if a science policy office is established in the White House by legislation, no president would be able to abolish it without first getting express approval from Congress.

Of more immediate concern, however, is the fact that it will take Congress several months to pass a bill.

Stever



David

PERHAPS the most comprehensive survey yet conducted of the effects of legalised abortion in the United States has just been published by the Institute of Medicine, an organisation which is part of the National Academy of Sciences. It offers strong evidence to support the oft-heard contention that liberalised abortion laws save many women's lives because they result in a larger proportion of abortions being conducted in proper medical surroundings, rather than in the offices of back-street practitioners or by self-induced means.

The basis of the survey was a comparison between data collected before and after an abrupt change in abortion laws in the United States, resulting from a ruling by the Supreme Court in January 1973. In a highly controversial decision, the court severely limited the ability of state governments to restrict abortions performed during the early stages of pregnancy.

While the number of legal abortions performed in the United States climbed by about 29,000 between 1972 and 1973, according to statistics collected by the federal government, the number of known abortion-related deaths declined from 128 in 1970 to 47 in 1973. Much of that decline, the committee suggests, can be attributed to a drop in the number of illegal abortions.

Although there are no reliable

Abortion survey

statistics on the numbers of illegal abortions being performed, the committee notes that a survey of data from the first year of New York's non-restrictive abortion law suggests that about 70% of the abortions obtained legally would otherwise have been obtained illegally.

The survey provides a wealth of information on the hazards associated with abortions performed at various stages of pregnancy.

- The risks of maternal death as a risk of abortions performed during the first trimester are low—about 1.7 deaths per 100,000 abortions. They are much lower than the risks associated with full-term delivery (14 deaths per 100,000 live vaginal deliveries) or with surgical removal of tonsils and adenoids (5 deaths per 100,000 operations).

- The risks associated with abortions performed during the second trimester are much higher—about 12.2 deaths per 100,000 abortions.

- Those risks, in fact, increase "not only from the first to second trimester, but also by each week of gestation", the committee suggests. At eight weeks or less, the mortality risk is less than 0.5 deaths per 100,000 abortions. Between 9

and 10 weeks, it is 1.7, at 11 to 12 weeks, it increases to 4.2 and by 16 to 20 weeks, it reaches more than 17 deaths per 100,000 abortions.

On the basis of those findings, the committee recommends that "laws, medical practices and educational programs should enable and encourage women who have chosen abortion to obtain it in the first three months of pregnancy".

As for the suggestion that liberalised abortion may become a substitute for contraception, the committee found the data to be limited, but they nevertheless "suggest that the introduction of non-restrictive abortion laws in the U.S. has not lead to any documented decline in demand for contraceptive services".

The committee notes that the 1973 Supreme Court decision has crystallised opposition to abortion in the United States, and that its report is likely to generate considerable controversy. In a classic case of understatement, for example, the committee accepts that since it does not discuss ethical issues or questions concerning the fetus, "this approach implies an ethical position with which some may disagree".

(The report, *Legalized Abortion and the Public Health*, No IoM 75-02, is available from the Institute of Medicine, 2101 Constitution Avenue NW, Washington DC 20418.)

October is generally regarded as the earliest that a bill could reach Ford's desk, but it is more likely to be early next year before Congress completes its deliberations. In that case, with the Presidential election looming in November and a good chance that a Democratic Administration will be elected, it will be difficult to persuade an able scientist to come to Washington for what could be a very short stint as science adviser.

Congress also has a chance to put its own stamp on the arrangement by specifying exactly what it should cover and how extensive it should be. Congress is likely, however, to give the President pretty much what he asks for, and allow him considerable leeway in deciding how the office should operate. For a White House policy office to have any effect, it must establish its own working relationships with other units in the Administration, and most

important, the President must be able to work with it.

The fact that President Ford has acknowledged the need for a central science policy office is generally regarded as a welcome first step, however. As Dr Edward E. David jun., President Nixon's last science adviser, put it last week, "we're making progress, but it's a long road to get to something that will perform a useful function". □

Soviet scene

from Vera Rich

THE President of the Soviet Academy of Sciences, Mstislav V. Keldysh, has retired at the age of 64, on the grounds of ill-health. Originally a mathematician, whose researches included potential theory, the theory of confluent elliptic equations, and the theory of functions of a complex variable, Keldysh later became associated with the development of the aircraft industry, and his appointment as a Vice-President of the Academy of Sciences of the USSR in 1960 and his promotion to the presidency in 1961 was widely

seen to be directly connected with the expansion of the Soviet space programme under Khrushchev. As President of the Academy, Keldysh was, perhaps inevitably, noted more for his extensive speeches on all possible scientific occasions than for any direct contributions to genuine research. During his presidency he received a number of foreign and Soviet honours, including membership of the Mongolian Academy of Sciences, the German "Academia Leopoldiana", honorary doctorates from the Universities of Wrocław, Budapest, and New Delhi, and the Lenin Prize (1967). It has been announced that he is to be succeeded

as President of the Academy by the relatively obscure figure of Academician Vladimir A. Kotel'nikov, who has been variously described as an astronomer and a radio-electronics engineer, four years older than his predecessor, although certain unofficial reports from Moscow suggest that this appointment was by no means a foregone conclusion. Academician Vladimir Kirillin, Vice-Chairman of the Council of Ministers of the USSR, was at one point a strong favourite for the post.

- The "Patrice Lumumba University for the Friendship of Peoples" in Moscow is preparing to celebrate its fifteenth anniversary. Founded originally

as the "University for Friendship among Peoples" in 1960, it was intended, according to a statement by Mr Sofronov, Secretary of the "Solidarity Committee for the Countries of Asia and Africa" made during a visit to the Indonesian National University on February 21, 1960, to be an instrument for the training of a national intelligentsia for the Afro-Asian and Latin-American countries.

During the 15 years of its existence, the University has expanded from a student body of some 300 housed in a former military academy to some 7,500 students (5,000 from 89 emerging countries and 2,500 Soviet students) with a modern campus of functional design.

According to the official figures, the University has an impressive record in terms of specialists trained and alumni now occupying prominent posts as statesmen, public figures and highly placed academic and industrial personnel in their home countries. Yet the whole purpose of the University still seems in question. Its foundation was clearly not entirely for altruistic motives. As early as 1923, Lenin was looking to what is now called the Third World as the decisive factor in the growth of World Communism (*Pravda*, March 4, 1923). He saw the problem primarily in terms of the countries of the Far East, but by the late 1950s it was clear that his theories could with equal validity be applied to Africa and Latin America. Clearly, the chance of educating students from these post-colonial countries in Moscow, rather than leaving them to follow the traditional path to London or Paris was an opportunity not to be missed. The question still remains, however—why a separate university, rather than scholarships at the ordinary universities of the Soviet Union? Since the only language of instruction at the Lumumba University is Russian the problem cannot be one of linguistic convenience, since the students have to learn Russian anyway before beginning their studies.

Perhaps the answer lies in the selection techniques. The Russian students who attend the Lumumba University seem to be very carefully selected to act as unofficial "tutors" to their foreign co-students. And the foreign students themselves are selected very carefully by the Russian authorities. This might seem reasonable, since after all it is the Soviet Union which is financing the University, but a number of countries, particularly those of South-East Asia, maintain that the selection procedure constitutes a breach of their own educational regulations, and have refused exit permits to students selected for the Lumumba University.

Whatever the reason, the Lumumba University students seem to be well isolated from the main body of Russian student life, even from that of the nearby Lomonosov Moscow State University. From time to time, complaints are voiced by former students about their feeling of isolation, which, in spite of the official title of the University, smacks to them of racial prejudice.

● As far as the Lumumba University is concerned, the Soviet Union jealously guards its privilege of selecting students for enrolment, as host and financier of the scheme. It is well known, however, that this principle does not operate in reverse nor in the higher academic echelons. When it is a matter of conferences or visiting lectureships outside the Soviet Union, then the wishes of the hosts are disregarded, and the invited speakers frequently fail to arrive and are replaced by some irrelevant substitute.

This practice has, in the past, been frequently tolerated by the hosts in the name of cultural exchange, although the cultural benefit derived from the presence of the substitute speaker may be minimal. Occasionally, however, a firm stand is made. In April, 1975, Dr William McGill, President of Columbia University, announced that he would not "receive or deal with any visitors from the Soviet Union" until the Moscow Sinologist, Vitalii Rubin, a long-standing member of the illicit Sunday seminar for *refusnik* scientists, is granted a visa to take up his two-year-old invitation as a visiting lecturer at Columbia University. Dr McGill also said that he would "use every effort I can muster to persuade my colleagues, voluntarily, to withdraw . . . from all forms of exchange with the Soviet Union" until Rubin received the desired visa.



A hundred years ago

A STRANGE case of poisoning is reported from Stettin. A gentleman had bought a hat in a shop there, and, after having worn it for one or two days, was troubled with unbearable headache; at the same time little ulcers formed upon his forehead, his eyes were inflamed, and the whole of the upper part of his head was much swollen. It was evident that these symptoms were caused by the hat, and upon examination by a chemist it was found that the brown leather in the inside of the hat was coloured with a poisonous aniline dye. It appears that inflammation is unavoidable when this dye is in contact with any part of the skin.

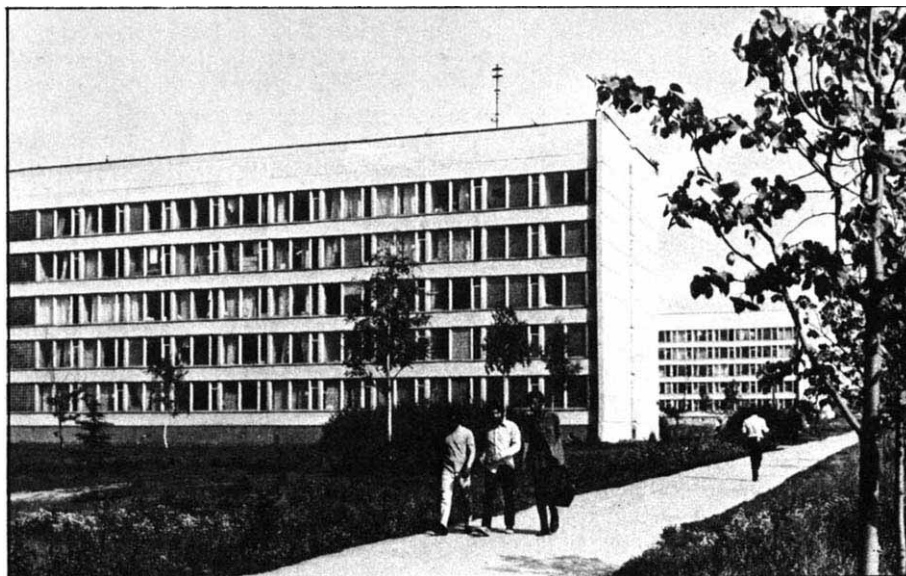
From *Nature* 12, 116; June 10, 1875.

● Census taking of natural phenomena—from polar bears to ant-hills—is a regular feature of the Soviet academic scene. The latest "census" to be announced by the Novosti agency, that of sea ice, seems, in fact, to consist of the processing of existing data, gathered by Soviet Arctic and Antarctic expeditions and photographs and other information from weather satellites.

According to these results, the average annual volume of floating ice is 38,700 km³, with an average daily accumulation/melt turnover of 37,000 km³, a process which, apparently, takes place twice as fast in the southern hemisphere than in the northern.

It is stated that the ice-cover of all parts of the world ocean is completely renewed every 11 years. No connection has so far been suggested between this figure and the sunspot cycle. □

Patrice Lumumba University



Fission wastes under glass

from a Correspondent

DOWN among the vineyards freshly laden with grapes, and the arid scrublands of Provençal France, environmental pollution is entering a new phase of test and contestation. The Phénix fast-breeder reactor, which went critical in late summer 1973 and fully operational last year, has now produced a full year's electrical supply of its net annual output of 233 megawatts (a trifle under one-hundredth the current yield of the entire French national power grid). Situated on the Rhône River at Marcoule in an atomic energy installation inaugurated in 1953, Phénix once again reminds both the public and specialists of nuclear energy's number-one nag: what happens to the radioactive wastes?

To help answer the question, and capitalising on a Canadian idea, Phénix's technical managers—the Commissariat à l'Energie Atomique and Electricité de France—have spent the past six years exploiting their PIVER process of vitrification. The idea is to treat and then store in a minimal volume, irradiated fuel residues in a stable and inert form. PIVER is a chemical incorporation, rather than merely a physical packaging, involving the fusion of waste materials with glass containing aluminium compounds, after the elimination of nitrogen oxides and various volatile products of fission.

The waste-glass amalgam is cast into blocks of 100 kg each, poured from Inconel stainless steel pots 25 cm in diameter and 2.50 m tall. The blocks are then stored on the spot in concrete-sheathed, vertical tubes 10 m deep. The process is said to make the waste material as insoluble as is chemically and physically possible, confine the radiation trapped inside (a batch of 12 vitrified tons confines 4 million curies), and provide good thermal stability during the early cooling and first 10–15 years of storage. PIVER regularly handles 14 tonnes of irradiated waste, in two semi-weekly operations. The vitrified blocks are eventually removed from their storage shafts to a half-buried, more isolated site.

But is this enough? Even the Commissariat's Yves Sousselier, a chemist, asks, "Will there always be radioactive wastes?" Recently, a group of forty professors from the Collège de France circulated a petition to the Parisian government calling for a halt to all nuclear energy activity. But the government is proceeding with plans to start work later this year on Super-Phénix, a 1,200 megawatt breeder reactor to be built further upstream along the Rhône at Creys-Malville. □

KENNETH MELLANBY



Treble recorder

CHILDREN in Britain and other western countries are larger and heavier than were individuals of the same age a hundred, fifty, or even only thirty-five years ago. This is usually considered to be a sign of better nutrition, though reports of obesity—perhaps our commonest form of malnutrition—are disturbingly common. But even without the disadvantage of fatness, is the present type of feeding doing good or harm? We know that rats which have an apparently suboptimal diet when young live longer than their littermates given a more liberal diet. It will be interesting to find whether the total life span of the present generation of children is longer or shorter than that of those who, for instance, were reared during the slump in the nineteen thirties, or who had the advantage of war-time rations.

It is generally believed that as well as growing faster, children become sexually mature earlier than they did in previous times. There are many publications which are believed to prove this point. All are based on observations on girls, and describe the age of the menarche in different communities. It is said that this was quite young (eleven or twelve) in the Middle Ages, that it rose to sixteen or more years, at least in Scandinavia, in the nineteenth century, and that it has since fallen steadily to a figure similar to that found three or four hundred years ago.

These results obtained from girls, are not universally accepted, for some of the records of the phenomenon on which they are based, derived largely from orphanage records, are somewhat suspect. Also I have heard the topic discussed by a group of women scientists who are now in their nineties; they considered that they and their school friends, towards the end of the last century, were as precocious as the present generation. However, it was pointed out that they came from comfortable middle class homes, and that

things might have been different among the ill-fed poor.

It always seems to me strange that there have been hardly any studies on boys, for here the onset of puberty could be so much more easily studied without the need for the sort of information which must have been hard to obtain among prudish Victorians. Some years ago I was involved in a pilot study of this subject, and I obtained the co-operation of Walter James, then editor of the *Times Educational Supplement*. I wished to find out whether there had been any change in the age at which boy's voices were observed to break. I thought it likely that we could find accurate records for this in the many choir schools attached to cathedrals and colleges in England.

The results of our study were, perhaps, surprising. We found that in the six schools investigated, there had been no significant change between the eighteen nineties and 1960. During the whole period boy's voices had broken, on an average, at a month or two after their thirteenth birthdays. Our preliminary figure suggested that there might have been a fall in this age by a few months in the seventy or so years covered by the investigation, but this was found to be a reflection of changes in the school system and not of the boy's physiology. At one time most choirboys remained at a choir school until the end of the session in which they ceased to sing treble; they now tend to be whisked off to a different school as soon as their voice breaks.

Our findings were briefly noted in Walter James' newspaper, and have received no notice from those concerned with this problem of allegedly earlier maturity. I think that the problem does warrant a much more exhaustive study. Discussion with school masters, particularly choir masters, suggests that there has indeed been little fall in the age of puberty of boys of all classes. This clearly has many sociological implications. But it does also mean that the female figures should be re-scrutinised. Both boys and girls have increased in height and weight in a parallel manner; it is strange that girls, apparently, should differ so much from boys in the change in the age of puberty.

● When I last referred to research contracts (May 1) I complained that they were often small and complicated to operate. In the majority of cases this is only too true. Unfortunately I gave the impression that the worst offenders might be found in the Department of Agriculture, Fisheries and Food. I am glad to be able to report that this is not so. Dr H. C. Pereira, the Chief Scientist, has been able to issue many sensible and simple contracts usually for very large sums. □

Asilomar conference on DNA recombinant molecules

At a meeting, held in California on February 24-27, an international group of scientists decided that strict controls should be placed on the use of an experimental technique which enables genes to be transplanted from one organism into another. This statement, drawn up by the organising committee for the conference is a summary of a report submitted to the Assembly of Life Sciences of the National Academy of Sciences and approved by its Executive Committee on May 20, 1975.*

This meeting was organised to review scientific progress in research on recombinant DNA molecules and to discuss appropriate ways to deal with the potential biohazards of this work. Impressive scientific achievements have already been made in this field and these techniques have a remarkable potential for furthering our understanding of fundamental biochemical processes in pro- and eukaryotic cells. The use of recombinant DNA methodology promises to revolutionise the practice of molecular biology. Although there has as yet been no practical application of the new techniques, there is every reason to believe that they will have significant practical utility in the future.

Of particular concern to the participants at the meeting was the issue of whether the pause in certain aspects of research in this area, called for by the Committee on Recombinant DNA Molecules of the National Academy of Sciences, USA in the letter published in July, 1974, should end; and, if so, how the scientific work could be undertaken with minimal risks to workers in laboratories, to the public at large and to the animal and plant species sharing our ecosystems.

The new techniques, which permit combination of genetic information from very different organisms, place us in an area of biology with many unknowns. Even in the present, more limited conduct of research in this field, the evaluation of potential bio-

hazards has proved to be extremely difficult. It is this ignorance that has compelled us to conclude that it would be wise to exercise considerable caution in performing this research. Nevertheless, the participants at the conference agreed that most of the work on construction of recombinant DNA molecules should proceed provided that appropriate safeguards, principally biological and physical barriers adequate to contain the newly created organisms, are employed. Moreover, the standards of protection should be greater at the beginning and modified as improvements in the methodology occur and assessments of the risks change. Furthermore, it was agreed that there are certain experiments in which the potential risks are of such a serious nature that they ought not to be done with presently available containment facilities. In the longer term serious problems may arise in the large scale application of this methodology in industry, medicine and agriculture. But it was also recognised that future research and experience may show that many of the potential biohazards are less serious and/or less probable than we now suspect.

Principles guiding the recommendations and conclusions

Although our assessments of the risks involved with each of the various lines of research on recombinant DNA molecules may differ, few, if any, believe that this methodology is free from any risk. Reasonable principles for dealing with these potential risks are: (1) that containment be made an essential consideration in the experimental design and, (2) that the effectiveness of the containment should match, as closely as possible, the estimated risk. Consequently, whatever scale of risks is agreed upon, there should be a commensurate scale of containment. Estimating the risks will be difficult and intuitive at first but this will improve as we acquire additional knowledge; at each stage we shall have to match the potential risk with an appropriate level of containment. Experiments requiring large scale operations would seem to be riskier than equivalent experiments done on a small scale and, therefore, require more stringent containment procedures. The use of cloning vehicles or

vectors (plasmids, phages) and bacterial hosts with a restricted capacity to multiply outside of the laboratory would reduce the potential biohazard of a particular experiment. Thus, the ways in which potential biohazards and different levels of containment are matched may vary from time to time particularly as the containment technology is improved. The means for assessing and balancing risks with appropriate levels of containment will need to be re-examined from time to time. Hopefully, through both formal and informal channels of information within and between the nations of the world, the way in which potential biohazards and levels of containment are matched would be consistent.

Containment of potentially biohazardous agents can be achieved in several ways. The most significant contribution to limiting the spread of the recombinant DNAs, is the use of biological barriers. These barriers are of two types: (1) fastidious bacterial hosts unable to survive in natural environments, and (2) non-transmissible and equally fastidious vectors (plasmids, bacteriophages or other viruses) able to grow only in specified hosts. Physical containment, exemplified by the use of suitable hoods, or where applicable, limited access or negative pressure laboratories, provides an additional factor of safety. Particularly important is strict adherence to good microbiological practices which, to a large measure can limit the escape of organisms from the experimental situation, and thereby increase the safety of the operation. Consequently, education and training of all personnel involved in the experiments is essential to the effectiveness of all containment measures. In practice these different means of containment will complement one another and documented substantial improvements in the ability to restrict the growth of bacterial hosts and vectors could permit modifications of the complementary physical containment requirements.

Stringent physical containment and rigorous laboratory procedures can reduce but not eliminate the possibility of spreading potentially hazardous agents. Therefore, investigators relying upon "disarmed" hosts and vectors for additional safety must rigorously test the effectiveness of these agents before accepting their validity as biological barriers.

*Paul Berg (Stanford), David Baltimore (MIT), Sydney Brenner (MRC, Cambridge), Richard O. Roblin III (Harvard) and Maxine F. Singer (NIH).

Recommendations for matching types of containment with types of experiments

No classification of experiments as to risk and no set of containment procedures can anticipate all situations. Given our present uncertainties about the hazards, the parameters proposed here are broadly conceived and meant to provide provisional guidelines for investigators and agencies concerned with research on recombinant DNAs. However, each investigator bears a responsibility for determining whether, in his particular case, special circumstances warrant a higher level of containment than is suggested here.

Types of containment

(1) **Minimal risk:** This type of containment is intended for experiments in which the biohazards may be accurately assessed and are expected to be minimal. Such containment can be achieved by following the operating procedures recommended for clinical microbiological laboratories. Essential features of such facilities are no drinking, eating or smoking in the laboratory, wearing laboratory coats in the work area, the use of cotton-plugged pipettes or preferably mechanical pipetting devices and prompt disinfection of contaminated materials.

(2) **Low risk:** This level of containment is appropriate for experiments which generate novel biotypes but where the available information indicates that the recombinant DNA cannot alter appreciably the ecological behaviour of the recipient species, increase significantly its pathogenicity, or prevent effective treatment of any resulting infections. The key features of this containment (in addition to the minimal procedures mentioned above) are a prohibition on mouth pipetting, access limited to laboratory personnel, and the use of biological safety cabinets for procedures likely to produce aerosols (for example, blending and sonication). Though existing vectors may be used in conjunction with low risk procedures, safer vectors and hosts should be adopted as they become available.

(3) **Moderate risk:** Such containment facilities are intended for experiments in which there is a probability of generating an agent with a significant potential for pathogenicity or ecological disruption. The principal features of this level of containment, in addition to those of the two preceding classes, are that transfer operations should be carried out in biological safety cabinets (for example, laminar flow hoods), gloves should be worn during the handling of infectious materials, vacuum lines must be pro-

tested by filters and negative pressure should be maintained in the limited access laboratories. Moreover, experiments posing a moderate risk must be done only with vectors and hosts that have an appreciably impaired capacity to multiply outside of the laboratory.

(4) **High risk:** This level of containment is intended for experiments in which the potential for ecological disruption or pathogenicity of the modified organism could be severe and thereby pose a serious biohazard to laboratory personnel or the public. The main features of this type of facility, which was designed to contain highly infectious microbiological agents, are its isolation from other areas by air locks, a negative pressure environment, a requirement for clothing changes and showers for entering personnel and laboratories fitted with treatment systems to inactivate or remove biological agents that may be contaminants in exhaust air, and liquid and solid wastes. All persons occupying these areas should wear protective laboratory clothing and shower at each exit from the containment facility. The handling of agents should be confined to biological safety cabinets in which the exhaust air is incinerated or passed through Hepa filters. High risk containment includes, in addition to the physical and procedural features described above, the use of rigorously tested vectors and hosts whose growth can be confined to the laboratory.

Types of experiments

Accurate estimates of the risks associated with different types of experiments are difficult to obtain because of our ignorance of the probability that the anticipated dangers will manifest themselves. Nevertheless, experiments involving the construction and propagation of recombinant DNA molecules using DNAs from (1) prokaryotes, bacteriophages and other plasmids, (2) animal viruses, and (3) eukaryotes have been characterised as minimal, low, moderate and high risks to guide investigators in their choice of the appropriate containment. These designations should be viewed as interim assignments which will need to be revised upward or downward in the light of future experience.

The recombinant DNA molecules themselves, as distinct from cells carrying them, may be infectious to bacteria or higher organisms. DNA preparations from these experiments, particularly in large quantities, should be chemically inactivated before disposal.

(1) **Prokaryotes, bacteriophages and bacterial plasmids:** Where the construc-

tion of recombinant DNA molecules and their propagation involves prokaryotic agents that are known to exchange genetic information naturally, the experiments can be performed in minimal risk containment facilities. Where such experiments pose a potential hazard, more stringent containment may be warranted.

Experiments involving the creation and propagation of recombinant DNA molecules from DNAs of species that ordinarily do not exchange genetic information, generate novel biotypes. Because such experiments may pose biohazards greater than those associated with the original organisms, they should be performed, at least, in low risk containment facilities. If the experiments involve either pathogenic organisms, or genetic determinants that may increase the pathogenicity of the recipient species, or if the transferred DNA can confer upon the recipient organisms new metabolic activities not native to these species and thereby modify its relationship with the environment, then moderate or high risk containment should be used.

Experiments extending the range of resistance of established human pathogens to therapeutically useful antibiotics or disinfectants should be undertaken only under moderate or high risk containment depending upon the virulence of the organism involved.

(2) **Animal viruses:** Experiments involving linkage of viral genomes or genome segments to prokaryotic vectors and their propagation in prokaryotic cells should be performed only with vector-host systems having demonstrably restricted growth capabilities outside the laboratory and with moderate risk containment facilities. Rigorously purified and characterised segments of non-oncogenic viral genomes or of the demonstrably non-transforming regions of oncogenic viral DNAs can be attached to presently existing vectors and propagated in moderate risk containment facilities; as safer vector-host systems become available such experiments may be performed in low risk facilities.

Experiments designed to introduce or propagate DNA from non-viral or other low risk agents in animal cells should use only low risk animal DNAs as vectors (for example, viral, mitochondrial) and manipulations should be confined to moderate risk containment facilities.

(3) **Eukaryotes:** The risks associated with joining random fragments of eukaryote DNA to prokaryotic DNA vectors and the propagation of these recombinant DNAs in prokaryotic hosts are the most difficult to assess.

A priori, the DNA from warm-blooded vertebrates is more likely to contain cryptic viral genomes poten-

tially pathogenic for man than is the DNA from other eukaryotes. Consequently, attempts to clone segments of DNA from such animal and particularly primate genomes should be performed only with vector-host systems having demonstrably restricted growth capabilities outside the laboratory and in a moderate risk containment facility. Until cloned segments of warm-blooded vertebrate DNA are completely characterised, they should continue to be maintained in the most restricted vector-host system in moderate risk containment laboratories; when such cloned segments are characterised they may be propagated as suggested above for purified segments of virus genomes.

Unless the organism makes a product known to be dangerous (such as toxin, virus), recombinant DNAs from cold-blooded vertebrates and all other lower eukaryotes can be constructed and propagated with the safest vector-host system available in low risk containment facilities.

Purified DNA from any source that performs known functions and can be judged to be non-toxic, may be cloned with currently available vectors in low risk containment facilities. (Toxic here includes potentially oncogenic products or substances that might perturb normal metabolism if produced in an animal or plant by a resident microorganism.)

(4) **Experiments to be deferred:** There are feasible experiments which present such serious dangers that their performance should not be undertaken at this time with the currently available vector-host systems and the presently available containment capability. These include the cloning of recombinant DNAs derived from highly pathogenic organisms (that is, Class III, IV and V aetiologic agents as classified by the United States Department of Health, Education and Welfare), DNA containing toxin genes and large scale experiments (more than 10 litres of culture) using recombinant DNAs that are able to make products potentially harmful to man, animals or plants.

Implementation

In many countries steps are already being taken by national bodies to formulate codes of practice for the con-

duct of experiments with known or potential biohazard*†. Until these are established, we urge individual scientists to use the proposals in this document as a guide. In addition, there are some recommendations which could be immediately and directly implemented by the scientific community.

Development of safer vectors and hosts

An important and encouraging accomplishment of the meeting was the realisation that special bacteria and vectors which have a restricted capacity to multiply outside the laboratory can be constructed genetically, and that the use of these organisms could enhance the safety of recombinant DNA experiments by many orders of magnitude. Experiments along these lines are presently in progress and in the near future, variants of λ bacteriophage, non-transmissible plasmids and special strains of *E. coli* will become available. All of these vectors could reduce the potential biohazards by very large factors and improve the methodology as well. Other vector-host systems, particularly modified strains of *Bacillus subtilis* and their relevant bacteriophages and plasmids, may also be useful for particular purposes. Quite possibly safe and suitable vectors may be found for eukaryotic hosts such as yeast and readily cultured plant and animal cells. There is likely to be a continuous development in this area and the participants at the meeting agreed that improved vector-host systems which reduce the biohazards of recombinant DNA research will be made freely available to all interested investigators.

Laboratory procedures

It is the clear responsibility of the principal investigator to inform the staff of the laboratory of the potential hazards of such experiments, before they are initiated. Free and open discussion is necessary so that each individual participating in the experiment fully understands the nature of the experiment and any risk that might be involved. All workers must be properly trained in the containment procedures that are designed to control the hazard, including emergency actions in the event of a hazard. It is also recommended that appropriate health surveillance of all personnel, including serological monitoring, be conducted periodically.

Education and reassessment

Research in this area will develop very quickly and the methods will be applied to many different biological problems. At any given time it is im-

possible to foresee the entire range of all potential experiments and make judgments on them. Therefore, it is essential to undertake a continuing reassessment of the problems in the light of new scientific knowledge. This could be achieved by a series of annual workshops and meetings, some of which should be at the international level. There should also be courses to train individuals in the relevant methods since it is likely that the work will be taken up by laboratories which may not have had extensive experience in this area. High priority should also be given to research that could improve and evaluate the containment effectiveness of new and existing vector-host systems.

New knowledge

This document represents our first assessment of the potential biohazards in the light of current knowledge. However, little is known about the survival of laboratory strains of bacteria and bacteriophages in different ecological niches in the outside world. Even less is known about whether recombinant DNA molecules will enhance or depress the survival of their vectors and hosts in nature. These questions are fundamental to the testing of any new organism that may be constructed. Research in this area needs to be undertaken and should be given high priority. In general, however, molecular biologists who may construct DNA recombinant molecules do not undertake these experiments and it will be necessary to facilitate collaborative research between them and groups skilled in the study of bacterial infection or ecological microbiology. Work should also be undertaken which would enable us to monitor the escape or dissemination of cloning vehicles and their hosts.

Nothing is known about the potential infectivity in higher organisms of phages or bacteria containing segments of eukaryotic DNA and very little about the infectivity of the DNA molecules themselves. Genetic transformation of bacteria does occur in animals suggesting that recombinant DNA molecules can retain their biological potency in this environment. There are many questions in this area, the answers to which are essential for our assessment of the biohazards of experiments with recombinant DNA molecules. It will be necessary to ensure that this work will be planned and carried out; and it will be particularly important to have this information before large scale application of the use of recombinant DNA molecules is attempted. □

*Advisory Board for the Research Councils. *Report of the Working Party on the Experimental Manipulation of the Genetic Composition of Micro-Organisms*. (HMSO Cmd 5880, 1975).

†National Institutes of Health Recombinant DNA Molecule Program Advisory Committee.

news and views

Virological hazards in routine procedures

from Robin Weiss

DURING the past year there has been much discussion about the ethics and potential hazards of 'genetic engineering', particularly on the splicing together of unrelated DNA fragments with the aid of specific restriction enzymes. The public debate was set off at the 1973 Gordon Conference on Nucleic Acids (Singer and Soll, *Science*, **181**, 1114; 1973) and by Ziff's article on "Benefits and hazards of manipulating DNA" in the *New Scientist* (24 October, 1973), and led to the announcement by Berg *et al.* (*Science*, **185**, 332; 1974 and *Nature*, **250**, 175; 1974) of a voluntary and temporary 'moratorium' on the manufacturing of certain kinds of DNA recombinants. In Britain, this stimulated the setting up of a government Working Party under Lord Ashby's chairmanship on the Experimental Manipulation of the Genetic Composition of Microorganisms. The Ashby committee, whose report was published earlier this year (HMSO, Cmnd 5880), suggested that the use of these recombinants should not be prohibited provided that it were subject to rigorous control in the laboratory; it further recommended that the safeguards of traditional containment procedures should be reinforced by genetical devices to 'disarm' the microorganisms used and that studies should be made on the viability in the human intestine of laboratory strains of *E. coli*, such as K12. Progress on disarming was reported at the International Conference on Recombinant DNA Molecules at Asilomar, California, three months ago, and in this issue of *Nature*, Anderson (p. 502) and Smith (p. 500) report that *E. coli* K12 will survive and multiply for a few days in the human intestine but that viability is low, as is the transfer of plasmids to the indigenous *E. coli* population.

Recently the government Working Party, under the chairmanship of Sir George Godber, on the Laboratory Use of Dangerous Pathogens published its report (HMSO, Cmnd 6054), which was reviewed in last week's issue of *Nature*. The working party, set up following the fatal transmission of smallpox virus from an accidentally infected laboratory worker to two outside contacts, recommends much more stringent control than has hitherto been imposed over known human and animal pathogens.

Despite the recent concern about genetic engineering and laboratory use of dangerous pathogens, there has been little or no public discussion about the possibility of unknown pathogens that might be unwittingly generated to quite high titres during routine procedures of experimental biology. I am thinking here not only of the possibility that laboratory variants of viruses could be more harmful than the wild-type isolates from which they were derived, but also of the activation and selection of RNA tumour viruses, particularly in tissue transplants in immunodeficient recipients and in somatic cell hybrids.

Xenografts and xenotropic viruses

When McAllister *et al.* (*Nature new Biol.*, **235**, 3; 1972) implanted cells of a human sarcoma line into the brain of a foetal cat and subsequently re-established the tumour in culture, the cells were found to be chronic producers of a C-type virus, named RD114. At first this virus was thought to be of human origin but later several laboratories showed that it represented an endogenous feline virus, unrelated to the better known, horizontally-transmitted feline leukaemia viruses. The RD114 virus grows to high titres in human cells, but feline cells do not support its replication. In a similar study, Todaro *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 859; 1973) obtained a murine C-type virus, AT-124, from human sarcoma cells inoculated into mice treated with anti-thymocyte serum. This virus also grows selectively in human cells but not in mouse cells. New Zealand Black mice spontaneously produce a similar virus which has been implicated in the auto-immune disease complex characteristic of this inbred strain (Levy, *Am. J. clin. Path.*, **62**, 258; 1974).

Levy has named these viruses, 'xenotropic', in contrast to the 'ecotropic' leukaemia viruses that can propagate infectiously in their host species of origin. Like some of the ecotropic viruses, xenotropic viruses appear to be transmitted in the 'resistant', natural hosts as Mendelian traits, probably representing integrated, DNA proviral forms of the viral genome. When activated to form

mature virus particles, these can only infect and replicate in host cells of certain foreign species. The implication of these findings is obvious, in view of the expanding use of xenografts of human tumour tissue in immunosuppressed and nude, athymic mice, but it has largely been ignored. Immunodeficient mice are routinely held in containment facilities for their own protection from infection, but human tumour cells subsequently explanted from such hosts might be handled without special precautions by laboratory workers who may be wholly unaware that the cells may be releasing C-type viruses infectious for human cells.

I am not aware of any studies to ascertain whether or not nude (*nu*) mice harbour xenotropic viruses, but this kind of virus is carried by many commonly used inbred strains, such as NIH-Swiss and BALB/c, and the *nu* gene can be cross-bred into any genetic background. It is possible that xenotropic virus is not activated in *nu* mice, though partial immune suppression as well as immune stimulation (graft-versus-host reactions and mixed lymphocyte reactions *in vitro*) appear to selectively activate such viruses (Scherr, Lieber and Todaro, *Cell*, **1**, 55; 1974). A study of the status of endogenous viruses in *nu* mouse strains should be carried out before they are used even more extensively than at present.

Many transplantable mouse tumours (Lieber *et al.*, *Int. J. Cancer*, **15**, 555; 1975) and cell lines in culture chronically produce ecotropic, xenotropic or both kinds of murine C-type virus. We do not know whether xenotropic murine viruses induce malignant growth, auto-immune disease or other diseases in susceptible hosts, and this question also demands early investigation. Rats and marmosets should serve as suitable hosts. Murine xenotropic viruses may prove to be innocuous for man, for mice and men have long lived together without apparent harm, but ignorance does not spell safety.

Cell fusion

All the potential hazards of endogenous xenotropic viruses raised already in relation to xenografts also apply to the use of somatic cell hybridisation tech-

niques, with the added hazard that inactivated Sendai virus, commonly used as an aid to cell fusion, can render cells susceptible to viruses to which they are normally resistant.

Neff and Enders (*Proc. Soc. exp. Biol. Med.*, **127**, 260; 1968) first showed that poliovirus, which does not infect hamster cells, will replicate in and kill hamster cells if introduced through alterations of the cell surface mediated by inactivated Sendai virus. This technique has also been used in several laboratories to overcome the genetic resistance of cells to infection with avian RNA tumour viruses and to mediate the infection of virus particles defective for envelope glycoproteins. In cell fusion experiments, the use of inactivated Sendai virus may cause the spread of latent viruses to the alternate host cell type. Stocks of Sendai virus used for cell fusion are prepared from large pools of infected chicken eggs that commonly contain congenitally transmitted avian RNA tumour viruses. The rate of inactivation of avian tumour viruses by ultraviolet light is some 20-fold lower than that of paramyxoviruses like Sendai (Levinson and Rubin, *Virology*, **28**, 533; 1966). Thus ultraviolet-inactivated Sendai virus stock distributed for cell fusion studies may frequently contain incompletely inactivated avian tumour virus, as I reported some years ago (*J. gen. Virol.*, **5**, 511; 1969). The avian tumour virus would not naturally penetrate mammalian cells but it will with the aid of inactivated Sendai virus; once introduced into a mammalian cell, the provirus formed may persist indefinitely in the cell's progeny as an inapparent infection (Boettiger, *Cell*, **3**, 71; 1974). β -Propiolactone rapidly inactivates RNA tumour viruses as efficiently as it inactivates Sendai virus, but this reagent is itself a dangerous and volatile carcinogen, and it must be handled with considerable caution.

Many of the cell types commonly used in cell fusion studies, L cells and murine lymphoma cells, spontaneously release C-type RNA viruses. Some of these viruses may be xenotropic, and xenotropic viruses may be activated in heterokaryons or hybrids, especially when 5-bromodeoxyuridine is used in the selection technique. Two recent papers on the expression of xenotropic viruses in interspecific hybrids between mouse and rat (Scolnick and Parks, *Virology*, **59**, 168; 1974) and between mouse and man (Gazdar, Russel and Minna, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2642; 1974) suggest that the murine xenotropic virus is repressed in the hybrid cells. But, the mouse-rat hybrids were permissive for the replication of a murine ecotropic virus and for simian (woolly monkey) sarcoma virus, although the parent rat and mouse cells

respectively were not susceptible to infection with these viruses. Therefore it should by no means be concluded that interspecific hybrids between resistant and susceptible species are resistant to virus replication. Indeed, cell fusion was used several years ago to illustrate the rescue of Rous sarcoma virus (Svoboda, Machala and Hlozaneck, *Folia Biol.*, **13**, 155; 1967) and SV40 (Watkins and Dulbecco, *Proc. natn. Acad. Sci. U.S.A.*, **58**, 1396; 1967) from cells containing but not replicating the virus.

On mixed infection, RNA tumour viruses undergo high frequency recombination between host range and other markers (Vogt, *Virology*, **46**, 947; 1974; Kawai and Hanafusa, *Virology*, **49**, 37; 1972) and recombination takes place between endogenous and exogenous viruses too (Weiss, Mason and Vogt, *Virology*, **52**, 535; 1973). New and hazardous recombinants could arise in interspecific hybrids. Phenotypic mixing between viral envelope components is an even more widespread phenomenon, giving rise to transient viral forms that may have a vastly extended host range (see, for example, Huang, Palma, Hewlett and Roizman, *Nature*, **252**, 743; 1974; Weiss, Boettiger and Love, *Cold Spring Harbor Symp. quant. Biol.*, **39**, 1974). Fusion of cells that may separately harbour different chronic or latent infections may thus give rise to unwanted hybrid viruses, and the laboratory worker may be unaware when this happens.

None of the points I have raised about heterospecific cell fusion or transplants constitutes a proven virological hazard; neither do those raised over the introduction of animal virus genes into bacterial plasmids or viruses. My aim is merely to show how complex the consequences of cell fusion can be. Ironically, the virological laboratories are probably the safest ones, because only in those will the appropriate tests for detection be carried out.

Infectious DNA from normal cells

Hill and Hillova (*Nature new Biol.*, **237**, 35; 1972) first demonstrated the rescue of proviral DNA of Rous sarcoma virus in mammalian cells by 'transfection' to permissive chicken cells. This technique has now been proven effective for the rescue of xenotropic endogenous proviruses in chick cells (Cooper and Temin, *Cold Spring Harbor Symp. quant. Biol.*, **39**, 1974) and in mouse cells (Scolnick and Bumgarner, *J. Virol.*, **15**, 1293; 1975). Simply by adsorbing DNA extracted from normal mouse tissues to human cells, the xenotropic viral genome might be incorporated and activated to replicate in the recipient cells. Thus normal cellular DNA of a much used experimental species constitutes a potential hazard

without incorporation into a plasmid or bacteriophage.

Are virus mutants dangerous?

The Working Party on the Laboratory Use of Dangerous Pathogens referred to already has recommended a severe reduction in the experimental use of pathogenic viruses in the UK. Laboratory viruses that have been favoured for molecular biology and genetics, such as Fowl Plague virus, Newcastle Disease virus and Vesicular Stomatitis virus are placed in the same category as Smallpox virus, Marburg virus and the Lassa fever agent, in requiring the most stringent controls possible, whereas the report makes no mention at all of tumour viruses. Clearly, this will seriously affect experimental animal virology in this country and it will be a difficult task to balance the restriction to scientific endeavour against the risk to economically important animals.

Here I wish to stress the element of risk to the health of the laboratory worker studying virus mutants and variants. We usually assume that virus mutants which are conditionally or non-conditionally defective in replication must be less pathogenic than their wild-type progenitors, but this may not invariably be the case. Defective polyoma virus in mice, for instance, is more oncogenic than the wild type (Defendi and Jensen, *Science*, **157**, 703; 1967). Huang and Baltimore (*Nature*, **226**, 325; 1970) first suggested that the defective, interfering (DI) particles that arise on high multiplicity passage of many animal viruses may play a role in limiting viral disease processes *in vivo*. Where unrestricted viral replication is lethal this may well be true, for Doyle and Holland (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 2105; 1973) have shown that administration of purified, homologous DI particles will considerably prolong the survival of mice inoculated intracerebrally with Vesicular Stomatitis virus. Instead of dying within 2 to 3 days of infection, the mice suffered a progressive, neural paralysis, which the authors named 'slow', though the mice began to die by 8 days post-infection! In some cases, excess DI particles inoculated with small doses of non-defective virus gave rise to genuinely slow, persistent infections. Now in mice the alternatives seem to be acute viraemia and early death, and persistent infection leading to paralysis. In man, however, the choice is more likely to be acute viraemia leading to recovery and immunity, and persistent infection leading to paralysis.

Similar arguments apply to the pathogenesis of temperature-sensitive (*ts*) virus mutants. Some *ts* mutants of reovirus (Fields, *New Engl. J. Med.*, **287**, 1026; 1972) and of measles virus (Haspel and Rapp, *Science*, **187**, 450; 1975) cause hydrocephalus and runting

in mice whereas the wild types kill the host quickly. A *ts* mutant of Vesicular Stomatitis virus paralyzes hamsters whereas the wild type causes acute death (Stanners and Goldberg, personal communication). These mutants may serve as valuable models of persistent neurological infections and slow virus disease in man. But the experimental mutants themselves may be dangerous.

These views are not intended to raise a hue and cry over 'callous and wicked' experimental biologists and it is to be hoped that the potential dangers alluded to will prove to be spurious. Nevertheless, it would be irresponsible not to bring these possible hazards to the attention of laboratory workers engaged in the biological manipulations discussed. This was not done in the two government reports and therefore no further reason is required for doing so here.

Pattern formation at EMBO

from R. S. Edgar

The First Annual EMBO Symposium on Developmental Genetics was held at Hirschorn, West Germany, from April 30–May 3.

ONE of the speakers remarked that when molecular biologists enter the field of development they find themselves rapidly retreating into the 18th century. I took him to mean that they find themselves wrestling with issues such as preformation and epigenesis (in modern guise, of course).

Histones

The meeting opened innocently enough with talks by E. Bradbury (Portsmouth Polytechnic) and R. Kornberg (MRC Laboratory of Molecular Biology, Cambridge) on the recent substantial advances made in understanding the role the histones play in determining the primary structure of the eukaryotic chromosome. Bradbury pointed out that the 120 Å repeat found for chromatin a number of years ago by Wilkins was due largely to the histones and could be best explained by a regular bead model with the DNA wrapped around a histone core. Kornberg provided evidence through careful nuclease digestion, chemical cross-linking and electron microscopic studies that the basic repeat structure was a tetramer bead consisting of one each of the histones H2A, H2B, H3 and H4. This bead protects 200 base pairs of DNA from nuclease digestion. Between the beads up to 20

base pairs of DNA are accessible to nuclease attack. This simple structure was apparently missed by earlier workers because of inadequate preparation procedures. Histone H1 is apparently not one of the basic bead histones—but Bradbury described some experiments implicating H1 in higher order packing of the chromosomes. (The packing ratio of the DNA in chromatin is about 7:1. In a metaphase chromosome, however, it is at least an order of magnitude greater, but the explanation for this is not yet known.)

Fifty years ago the nature of the gene was a lively question. Although largely settled now for prokaryotic organisms, this question is still open for the eukaryotes. In *Drosophila* the available evidence points to one band of the salivary chromosome as delimiting the extent of a gene. But the organisation of DNA in terms of structural and regulatory elements within this region remains to be resolved. A. Chovnick (University of Connecticut) summarised the state of his studies on the structure of the *rosy* locus in *Drosophila*, which controls the structure of the enzyme xanthine dehydrogenase and determines eye colour. He found that mutations affecting the structure of this enzyme in terms of electrophoretic mobility changes and also other putative missense mutations (mutants manifesting intragenic complementation) all map within 5×10^{-3} map units. This region is estimated to cover 4,000 nucleotide pairs of DNA, not grossly out of line with the number needed to code for xanthine dehydrogenase. Surprisingly, all the mutants that fail to make detectable protein also seem to map within this region. One might expect that some such mutants would be affected in regulatory elements. But Chovnick also reported the recent finding of mutants with alterations in the amount of enzyme made and also in the pattern of enzyme formation at different stages in development. One such allelic difference which affects the amount of enzyme formed has been found to be *cis* acting (and thus a possible operator-like element) and maps 5×10^{-3} map units away from the left boundary of the cluster of structural mutations. This result suggests the possibility that genetic analysis of regulatory elements in at least one gene of *Drosophila* is in the offing.

Although a number of the presentations at the meeting dealt with attempts to understand the molecular events that lead to the differentiated state in cells, the major focus of the meeting seemed to be on the earlier and more enigmatic aspects of development. How is pattern established in organisms and what is the nature of the determined state?

Compartments

Significant new information has been obtained about the mode of development of the adult structures from the imaginal disks of *Drosophila*. This work was described first by P. Lawrence (MRC Unit of Molecular Biology, Cambridge) and then by A. Garcia-Bellido (CSIC, Madrid, Spain). The various organs in the fly develop from small groups of cells which form early in development and subsequently proliferate, mainly during pupation. It has been found that development proceeds by the successive creation of 'compartments' and of compartments within compartments. These compartments have precisely defined boundaries across which cells do not migrate, thus any compartment may be thought of as a collection of founding cells (not necessarily of common lineage) whose descendants form the structures appropriate to that compartment. Using genetic mosaic analysis to trace clonal pedigrees, Garcia-Bellido has shown that for the dorsomethoracic (wing) segments anterior and posterior compartments are formed before hatching. A wing sub-compartment is then formed during the first larval instar and dorsal and ventral wing sub-compartments are formed about the same time.

Although the successive formation of compartments and sub-compartments progressively delimits the fate of cells, development and differentiation within a compartment is for a time at least regulative. Mosaic analysis indicates no reproducible topographical patterns of clones within compartments and clones of rapidly growing cells (clones of wild-type cells in flies of *minute* genotype) can outgrow the *minute* cells and nearly fill a compartment and still generate a normal differentiated pattern for that compartment. It appears that the homeotic mutants of *Drosophila*, known for many years, affect the genes which control development within compartments. The mutation *engrailed*, for example, acts to convert the posterior wing compartments into a mirror-image of the anterior compartment. Apparently the wild-type allele of the *engrailed* gene affects cell recognition and sorting properties during the creation of the anterior and posterior compartments since in the mutant, but not the wild type, cell mixing, as shown by clonal analysis, occurs across the boundary. A variety of such homeotic mutants are known and their properties fit well with the notion that these genes determine the fate of cells within compartments.

Insects have long been considered archetypal representatives of mosaic as opposed to regulative development. In insects the first cells that form in the blastoderm are thought each to be a progenitor of a specific and different

part of the adult structures. In contrast, the vertebrate egg is considered highly regulative since the whole organism may be reconstituted from any single descendent cell, at least through the first several divisions of the egg. That the distinction between mosaic and regulative development has been overdrawn was argued by K. Sander (Albert-Ludwig's-Universität, Freiburg). He presented experiments which demonstrated convincingly that in insects the early developmental stages are to a degree still regulative. In the leaf hopper, if material from the posterior pole of the egg is moved anteriorly the fate of cells forming in adjacent regions of the blastoderm can be altered. When the eggs of blowflies are pinched by ligation the fate of cells adjacent to the ligated region is altered. If the egg is closed off in the middle, the anterior portion will generate only the anterior segments 1, 2, 3 and 4 while the posterior portion forms only segments 7, 8, 9 and 10. The central segments do not form. However, asymmetrical ligations can generate those segments (for example, 1, 2, 3 and 6, 7, 8, 9 and 10). These results are most readily interpreted by assuming that an inducer gradient exists within the egg which is re-established after ligation and that the fate of cells in the central region is determined by the re-established gradients. Sander argued that this pre-pattern formation which gives rise to the determined blastoderm occurs late in the primitive insects and with evolution is projected back into the oocyte giving rise to the so-called mosaic developmental pattern where each cell that arises in the young embryo seems to have a defined fate.

Pattern formation

P. Sengal (University of Grenoble, France) summarised his elegant studies on pattern formation in the skin of amniotes. This was one of the few presentations at the meeting dealing with vertebrate development. Whereas the epidermis is the sole participant in forming such major surface features as hair, feathers and scales, the dermis plays a crucial role in determining the topographical pattern of these elements. Sengal was able to demonstrate the role these two tissues play in differentiation by culturing together epidermis and dermis from different sources (mouse, chick, duck and lizard) on chick allantoic membrane. In all cases the epidermis initiated development of structures appropriate to its ancestry independent of the origin of the underlying dermis but the pattern of epidermally-derived elements within the epidermis seemed to be controlled by the dermis. Sengal found that dermis from the mouse upper lip (which nor-

mally forms large and small hairs) induced the formation of large and small placoids in lizard epidermis and large and small feather follicles in chick epidermis, whereas mouse dorsal epidermis (which normally forms small hairs only) induced a dense pattern of small scales or feathers in the appropriate epidermis. Various combinations gave essentially the same answer—pattern information is transferred from

the dermis to the epidermis even between mouse and lizard tissues. But although gross pattern information can apparently be transmitted between tissues derived from animals of different classes, the structures formed by the epidermis in these conditions resemble only the early stages of differentiation of these elements. Apparently further specific instructions concerning the nature of the hair or

Thermal environment of plants

from Peter D. Moore

THE annual report of the Department of Plant Biology of the Carnegie Institution can usually be relied upon to supply stimulating ideas and information in the field of plant ecophysiology and the recently issued report for 1973–74 is no exception. Of particular interest is a series of field and laboratory experiments relating to the influence of the thermal environment upon the growth and development of several plant species.

The field experiments are reported by Björkman *et al.* (*Carnegie Inst. Yb.*, **73**, 748; 1974) and concern transplantation into two experimental 'gardens', one in Death Valley, a desert site with high temperatures and low rainfall (less than 40 mm) and the other at Bodega Head on the coast of California, where conditions are cool and humid (annual rainfall is 750–1,000 mm). A total of 17 species were used in transplant experiments, including some species of cool, oceanic climates and some from desert environments; many belong to the family Chenopodiaceae, including species of both C_3 and C_4 type (see *Nature*, **252**, 438; 1974). In Death Valley all died during the summer if irrigation was withheld. When supplied with water, however, eight of the species tested were capable of survival, but grew better between October and May than they did in full summer (June to September). One species, *Tidestromia oblongifolia*, not only survived at Death Valley when irrigated, but actually grew better in high summer than in the October to May period. All species except one of the cool, oceanic group failed to survive at Death Valley even when they were irrigated.

At the oceanic site, Bodega Head, irrigation had a far less marked effect and all species except one survived. The exception, once again, was *T. oblongifolia*. The experi-

ments demonstrate the importance of temperature as a determinant of survival and growth under conditions of ample water supply in these species and they also indicate that adaptations which permit growth at one climatic extreme may preclude survival under less severe conditions.

Transplant experiments have certain disadvantages. Failure to survive at a site may be due to such pressures as predation or parasitism rather than to an adverse physical environment. Björkman and his colleagues have met this objection by conducting controlled environment chamber experiments on four species representative of the various responses observed in the field. The results obtained parallel the field observations. *T. oblongifolia* failed to sustain its growth under a 16 °C (day)/ 11 °C (night) thermal regime whereas it doubled its dry weight in three days when grown at 45 °C (day)/ 31 °C (night). These conditions reflect those often experienced at Bodega Head and Death Valley, respectively. The oceanic species again grew better under cooler conditions.

Growth differences between species and between temperature regimes cannot be explained simply in terms of differential translocation of photosynthate because growth analysis experiments showed a similar allocation of material to root, shoot and leaf in all species under both sets of conditions. So temperature must act directly upon metabolic processes, perhaps upon membrane or enzyme stability. The authors also draw attention to the fact that *Tidestromia* is a C_4 plant, as are some of the oceanic types, such as *Atriplex sabulosa*. The experiments thus confirm the association of the C_4 mechanism with adaptation to high temperature environments, but also show that some C_4 plants have growth optima at relatively low temperatures.

feathers is required but is not provided by the heterologous dermis. Combinations of chick epidermis with duck dermis form proper feathers which resemble those of the duck in certain major features. Similarly mouse epidermis can be induced to produce hairs patterned by chick dermis if dermal cells from the palm (planta) of the mouse are interspersed between the mouse epidermis and chick dermis even though the planta dermis cannot by itself produce hair formation in mouse dermis. Apparently a previous pattern message is required before specific instructions concerning the kind of structures to be formed is transmitted from the dermis to the epidermis. These results suggest a hierarchy of determinative events, events which for me were reminiscent of the behaviour of homeotic genes in *Drosophila* as described by Garcia-Bellido and by Lawrence.

Molecular and cellular basis

None of the systems described provide any insight as to the molecular or cellular basis of the patterning and determination events. The work presented by K. Illmensee (Institute for Cancer Research, Philadelphia) gave perhaps the closest look at what is involved at the cellular level. In the *Drosophila* embryo the four most posterior pole cells which are set aside during blastoderm formation proceed to form the gonad of the mature animal. Illmensee was able to show that this determination event is induced by cytoplasmic determinants localised in the posterior pole cytoplasm and that they appear at this specific location quite early in the development of the oocyte. This was demonstrated by withdrawing posterior pole cytoplasm from an egg (or oocyte), injecting it into another region of the embryo and letting development proceed to the blastoderm stage. The future fate of cells forming in the region of the injection was tested by transplanting them to the posterior portion of another embryo, suitably marked genetically, and testing the host animal for gonadal mosaicism and also by following the manipulations by electron microscopy. Characteristic cytoplasmic granules were found in the pole cytoplasm; later, similar granules were found in the nuclei of the presumptive gonadal cells. But no causal connection between the granules and the determination event was asserted.

While these findings fit well the expectations of the Preformationists—that highly specific and irrevocable determination events occur early in insect development—the Epigeneticists might argue that the setting aside of the germplasm early in development is a special case and requires special

mechanisms. Indeed, in all of the other systems described, whether insect or vertebrate, it appears that pattern formation is preceded by the creation of a pre-pattern of some sort which determines the future fate of cells and their descendants, not in terms of specific differentiation, but in terms of general pattern decisions such as anterior, posterior or large and small. Thus the specific fate of individual cells is progressively defined rather than predetermined.

This epigenetic view received little support from the analysis of the development and anatomy of the ventral nerve cord of the nematode *Caenorhabditis* presented by S. Brenner (MRC Laboratory of Molecular Biology, Cambridge). This nematode has a ventral nerve cord which serves to innervate the body muscles. The dorsal muscles are innervated by a dorsal nerve cord which consists of fibres derived from cells located in the ventral nerve cord. This cord is quite simple in basic plan, consisting of 58 neurones of only eight types as defined by their anatomy and by the connections they form with other cells.

The newly-hatched worm, however, has a ventral cord consisting of only 15 neurones, the other 43 are added to the cord shortly after through the multiplication, migration and differentiation of nine neuroblasts. These events have been painstakingly followed in living animals. The pattern of division, migration and final fate of these cells is invariant and includes the death of certain cells in certain lineages.

These results are quite readily accounted for by a high degree of intracellular pre-programming of development and differentiation. Surgical experiments with a laser (in progress) may give more insight into the degree to which nematode development is regulated, if it is at all.

As Francis Crick (MRC Laboratory of Molecular Biology, Cambridge) remarked in his summary (to nobody's surprise) there is a very long way to go in explaining development in molecular and cellular terms. He also noted that except for the studies of homeotic mutants, the genetic analysis of development was curiously absent, and this in a meeting entitled Developmental Genetics.

Cropping natural animal populations

from Robert M. May

INTEREST in game ranching of natural animal populations as a food source goes back a long way. This interest is spurred by observations which suggest, for example, that in East Africa standing crops of conventional livestock

(mostly cattle) run around 32,000 pounds per square mile on moderately well-managed grassland, and around 16,000 pounds per square mile on Masailand savanna, whereas wild game runs around 90,000 pounds per square mile on similar savanna (see, for example, Watt, *Ecology and Resource Management*, 70–72; McGraw-Hill, 1968).

Journals such as the *Journal of Wildlife Management* or the *East African Wildlife Journal* contain numerous studies of the detailed population dynamics of species that are possible candidates for cropping. Given these data, the problem is to find the management strategy that will give the optimum sustainable yield. Two lines of attack may be distinguished. The first adopts some nonlinear or 'density dependent' model to describe the overall population dynamics (usually without age structure), and then uses econometric techniques to calculate the strategy which, under given assumptions, will maximise profits (for example, Clark, in a chapter in *Some Mathematical Problems in Biology*, Springer Verlag, 1974). The second constructs density independent 'actuarial tables' of age-specific survivorship and fecundity for the population, and then applies standard demographic techniques to find its intrinsic capacity for population growth and thus to estimate the sustainable yield (for example, Caughley and Birch, *J. Wildl. Mgmt*, **35**, 658–663; 1971).

These approaches are useful first steps, but neither is conducive to finding the best age-specific cropping strategy. Beddington (*J. appl. Ecol.*, **11**, 915–924; 1974, and also Beddington and Taylor, *Biometrics*, **29**, 801–809; 1973) has recently proved a surprisingly general and elegant theorem which sheds light on this question. He shows that, for an age-structured population of constant size, the sustainable yield is maximised by the complete removal of one age class, effectively reducing the age of maximum longevity of the population, and a partial removal of a second age class. No other age class is touched. These two age classes depend, of course, on the demographic details; Beddington provides a simple algorithm for finding which two.

The basic theorem also applies if the crop to be maximised is biomass, or some sort of economic value, rather than simply numbers of animals. The analysis may be extended to find the optimum sex ratio, under various biological and economic assumptions. It remains true that, for either sex, at most two age groups should be cropped: one partially and the other completely.

It is reassuring to note that these formal results accord with many traditional agricultural practices. For

instance, in hill sheep farming a proportion of male and female lambs are removed, and at a certain age all ewes are removed; the ratio of males to females is kept constant, and at another age all males are removed.

The practical application of these strategic insights may be hindered by difficulties in determining the animals' ages. And there are situations where highly age-specific cropping would not be feasible, even were the ages known accurately. This may explain why Beddington's results were not derived earlier in the fisheries literature: in other respects, the theory of optimal management of fisheries seems much more developed than that of game ranching, for obvious economic reasons.

For a wide variety of theoretical assumptions as to survivorship and fecundity curves, Beddington's algorithm suggests that the maximum sustainable yield is obtained by cropping all the animals of an age somewhat beyond that of peak fecundity, along with a fraction of those in the youngest age class. The ensuing survivorship curves are strikingly similar to those found in many natural populations of higher animals (as reviewed, for example, by Caughley, *Ecology*, **47**, 906-918; 1966). There is currently a burgeoning literature which seeks to understand the evolution of life history strategies (for example, Goodman, *Am. Nat.*, **108**, 247-268; 1974; Schaffer, *Am. Nat.*, **108**, 783-790, 1974; Southwood *et al.*, *Am. Nat.*, **108**, 791-804, 1974); although Beddington's work was done with the practical aim of studying exogenous manipulation of animal populations, it is plausible to speculate that his nonlinear optimisation techniques may eventually be bent to a more general discussion of the way endogenous evolutionary forces shape survivorship curves.

Rotating superfluid film?

from P. V. E. McClintock

If one rotates a metal disk coated with a thin adsorbed film of superfluid helium, does the helium move with the disk or does it remain at rest? This is the intriguing question to which Vittoratos and Meincke of Toronto University have recently been addressing themselves and, in contrast with earlier workers, they seem to have been successful in establishing at least part of the answer (*Phys. Rev. Lett.*, **34**, 796; 1975).

Common sense seems at first sight to suggest that the helium film should move with the metal substrate with

which it is in such intimate contact. On the other hand, if the helium is really superfluid then, by definition, there will be no frictional mechanism through which it can be brought into motion. There are, in addition, compelling theoretical reasons for believing that superfluid helium is actually incapable of rotating in a conventional way so that, even if an initially normal rotating film were cooled through the superfluid transition temperature at 2 K, one would not expect to end up with a rotating superfluid film.

The experimental chamber used at Toronto contained a capacitance cell formed from a pair of closely spaced aluminium plates 6 cm in diameter. It was anticipated that any rotation of the helium film adsorbed on the plates would result in a change of its thickness, thus leading to a change in capacitance because of the altered amount of dielectric between the plates. In fact, there were three separate capacitances each having the upper aluminium plate as a common element, the other elements being three small electrically isolated aluminium plugs mounted flush with the lower plate. One of these was positioned on the axis of rotation, and the others at radii of 1.4 and 2.0 cm from the axis. It was thus possible to measure the thickness of the film at these three positions with a resolution of about 2 Å. The films were prepared by condensing an appropriate quantity of gaseous helium into the cell so that, by carefully monitoring the capacitances, a film of any desired thickness up to about 300 Å could be established.

When the cell was rotated slowly it was found that the film was thinned to an equal extent at all measuring positions, thus demonstrating that the film itself was not rotating: if it had been, then the degree of thinning would have been different at different radial posi-

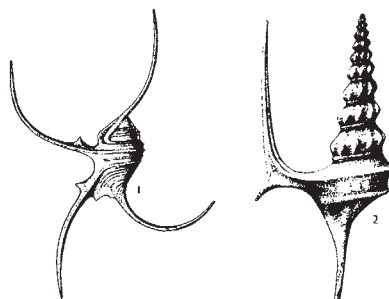
tions because of the centrifugal contribution to the potential energy of the adsorbed helium atoms (the overall uniform thinning indicating merely that there were regions within the rather complicated structure of the chamber, outside the capacitance cell, where the helium was in fact moving). When the angular velocity of the chamber reached a certain critical value, however, a difference in thickness developed abruptly between the film at 2 cm and that on the axis—suggesting that the film at 2 cm had come suddenly into some sort of rotational state.

Now, in the case of bulk liquid helium it has been well established that the superfluid does not rotate in a conventional manner. Instead, the liquid in a rotating vessel becomes threaded with vortex lines, each consisting of a linear singularity or core around which the superfluid flows with a tangential velocity which is inversely proportional to the radial distance from the core. The net movement of the superfluid arising from the presence of the dense array of vortex lines generated within a rapidly rotating container does, in fact, approximate to that of normal solid-body rotation.

Vittoratos and Meincke have concluded from a detailed analysis of their experimental results that a somewhat similar picture is applicable to the helium film on a rotating substrate. They believe that the situation can be understood in terms of a critical radius r_c from the axis of rotation at which the tangential velocity of the substrate is equal to the critical velocity v_c for the onset of dissipation in a flowing film of the same thickness. Outside r_c there is a dense array of vortex lines which, by lying parallel to the rotational axis, permit an effective rotation of the superfluid at approximately the same angular velocity as the substrate; but inside r_c there are no vortices at all. On a rotating substrate, according to their proposed model, there should always be, around the axis of rotation, a tranquil vortex-free region of the film whose radius varies inversely with the angular velocity of the container.

This makes an interesting contrast with the situation in a rotating container of bulk liquid helium where it is a critical angular velocity which matters, and where the first vortex line is found on the axis of rotation. Another major difference between two- and three-dimensional rotation seems to be in the criteria for vortex formation. In bulk liquid the critical angular velocity may be computed by minimising the energy of the liquid as a whole, whereas, in the film, the first vortex is apparently formed when the tangential velocity of the fastest moving part of

Mesozoic fossils



Left, *Tessarolax fittoni* from the Lower Greensand, Isle of Wight; right, *Anchura carinata* from the Gault in Kent. (From *British Mesozoic Fossils*, page 167; British Museum (Natural History), fifth edition, 1975.)

the substrate exceeds the linear critical velocity (v_c) of the film. There is, as yet, no theoretical explanation of these differences.

Although many features remain to be investigated in further more precise experiments—it would, for example, be useful to determine accurately the extent to which the angular velocity of the film outside r_c lags behind that of the substrate—it is evident that this work has provided the first useful insight into the rotational behaviour of a two-dimensional superfluid.

Chemists and physicists in resonance

from J. A. S. Smith

When Ponce de Leon first landed on the Atlantic coast of Florida on April 3 1513, he is said to have been seeking "a never failing spring of such marvellous efficacy that when the water is drunk, it makes old people young again". The 105 participants at the 3rd International Symposium on Nuclear Quadrupole Resonance Spectroscopy who assembled in Tampa, Florida, just 462 years and 11 days later, were not perhaps expecting such a dramatic change in their lives, but in any event they almost certainly experienced a scientific rejuvenation.

ONE common theme of a number of papers was the investigation of static and dynamic critical phenomena at phase transitions. Quadrupole and magnetic resonance studies of frequencies, line-widths, and relaxation times now provide an important method of study, and the expected cusp (both maximum and minimum) in the relaxation rate, T_1^{-1} , as a function of temperature was shown to occur in a number of instances, such as ^{23}Na relaxation in NaNO_2 and ^{75}As relaxation in $\text{NH}_4\text{H}_2\text{AsO}_4$ (F. Borsa and A. Rigamonti, University of Pavia). In related papers, R. L. Armstrong (University of Toronto) showed the importance of anharmonicity effects in the perovskite CsPbCl_3 by combining ^{35}Cl quadrupole resonance studies with neutron inelastic scattering spectroscopy, and H. Chihara (University of Osaka) presented further examples of displacive processes in the phase transitions observed in chloranil and ferroelectric $\text{NH}_4[\text{H}(\text{ClCH}_2\text{COO})_2]$. The theme was continued in a number of contributed papers concerned with such diverse structures as $[\text{CH}_3\text{NH}_3]_2\text{PtCl}_6$ (D. Nakamura *et al.*, Nagoya University), SbCl_3 (R. J. C. Brown *et*

al., Queen's University, Kingston, Ontario), NaSH (K. R. Jeffrey, Queen's University), and NaNO_2 and NaNbO_3 (A. Avogadro *et al.*, University of Pavia). A related topic of much current interest is that of molecular motion and relaxation: A. Tzalmuna (Hebrew University of Jerusalem) reviewed his applications of the sudden-jump approximation to such studies. Using ^{14}N quadrupole resonance, Y. Abe and his coworkers (Tokyo University of Education) demonstrated the presence of at least two molecular modes in ethylenediamine, in one of which the molecule twisted between two equivalent *gauche* configurations; T. A. Scott *et al.* (University of Florida, Gainesville) discussed the librations of the nitrogen molecule in both the pure solid and *p*-quinone clathrate; the motions of the PCl_3 group (I. G. Shaposhnikov *et al.*, Perm University, USSR) and $-\text{BCl}_3$ and $-\text{CCl}_3$ groups (S. Ardjomand and E. A. C. Lucken, University of Geneva) were also discussed in a number of molecules. E. Schempp (University of Pittsburgh) mentioned several crystals in which the acoustic phonon branches appear to dominate the negative temperature dependence of the frequencies at low temperatures, giving rise to a T^4 dependence.

The calculation of nuclear quadrupole interactions and electric field gradients in solids was reviewed by T. P. Das (State University of New York, Albany); in non-cubic metals, pseudo-potential calculations have been applied in recent years with limited success to evaluate the contribution of the conduction electrons. An important conclusion of these calculations is that the electric field gradient depends on the whole electron distribution, not just on those electrons near the Fermi surface which dominate the Knight shift. With solids such as Te, Se and S, tight-binding wave functions built out of atomic orbitals on the atoms need to be used, and even in non-cubic ionic crystals, recent work has shown the importance of allowing for the distortion of electronic orbitals by neighbouring ions through overlap and covalent bonding effects.

On the experimental side, the increasing importance of pulse techniques was evident in many papers. Among these, we may refer to the success of double resonance methods; R. Blinc (Institute J. Stefan, Ljubljana, Yugoslavia) reviewed very thoroughly these techniques and demonstrated that their sensitivity was now sufficient to detect ^{14}N quadrupole resonance of the peptide nitrogens in polyglycine. J. L. Ragle (University of Massachusetts, Amherst) discussed the elegant experimental methods by which he has detected ^2H quadrupole resonance with

good resolution (<1 kHz) in many halogenated hydrocarbons, with a sensitivity sufficient to detect deuterons 5 to 6 Å away from the marker nucleus (for example the β -deuterons in the pyridine ring of $\text{C}_5\text{D}_5\text{N} \cdot \text{CDCl}_3$). Other workers reported the double resonance detection of ^{55}Mn quadrupole resonance in carbonyls and ^{14}N quadrupole resonance in transition-metal glyoxime and bipyridyl complexes (T. L. Brown *et al.*, University of Illinois, Urbana), and ^{27}Al quadrupole resonance in AlBr_3 (N. Weiden and A. Weiss, Technische Hochschule, Darmstadt). Another new development is the revival of interest in high-pressure studies; in the alkylammonium halides (G. Jugie and J. A. S. Smith, University of London), chlorocyclophosphatrienes (W. H. Dagleish and A. L. Porte, University of Glasgow) and trihalides of As and Sb (G. C. Gillies and R. J. C. Brown, Queen's University), the observed pressure shifts were related to changes in the intermolecular bonding.

In chemical applications of the technique, there is clearly a tendency to widen the range of nuclei that are studied. We have referred earlier to the deuteron, ^{27}Al , ^{55}Mn , and among others we may mention ^{51}V studies of V_2O_5 (V. A. Gubanov *et al.*, Ural Science Centre, Sverdlovsk) and the attempts now being made in several laboratories to study the structure of organometallic compounds by quadrupole resonance, for example ^{187}Re resonance in trimethylsilyl and trimethylgermyl perhenates (H. Schmidbaur *et al.*, Technische Universität, Munich), ^{59}Co resonance in dicobalt octacarbonyl and its alkyne derivatives (M. C. L. Gerry *et al.*, University of British Columbia, Vancouver), and $^{69,71}\text{Ga}$, ^{115}In studies of group III organometallic compounds (T. B. Brill *et al.*, University of Delaware, Newark).

The widening range of problems now being studied by nuclear quadrupole resonance and relaxation was very evident, which is perhaps a consequence of the close liaison which exists between chemists and physicists in this branch of radiofrequency spectroscopy. The Florida symposium did much to forward this collaboration.

High pressure living

from A. L. Rice

HYDROSTATIC pressure increases in the sea by one atmosphere for every 10 metres or so in depth, so that in the deepest parts of the oceans pressures well in excess of 1,000 atmospheres prevail and almost nine tenths of the ocean bed experience pressures of 100 atmospheres or more. Until a little over a century ago these stupendous pressures, along with the other 'inhospit-

able' features of the deep-sea environment such as total darkness and low temperatures, were generally thought to preclude the existence of any life deeper than a few hundreds of metres. The great oceanographic voyages of the late nineteenth century dispelled this idea, for they demonstrated the existence of a rich fauna even at abyssal depths and stimulated an interest in the effects of high pressures on marine organisms, beginning with the classic works of Regnard and Certes in the 1880s and continuing, with a considerable upsurge in recent years, to the present day.

The instrumentation for this type of work is fraught with difficulties and for obvious technical reasons experiments on macroscopic organisms have, until fairly recently, been rather simple, usually being limited to the determination of lethal pressures for shallow-water animals. Nevertheless, a good deal of interesting information has been obtained. For one thing, although most of the shallow forms are killed by even short exposures to pressures much lower than those obtaining in the deep ocean trenches, many of them show a remarkable tolerance of pressures far outside their normal range of experience; common mussels (*Mytilus edulis*), for instance, may survive an hour's exposure to 800 atm. Second, the most pressure-tolerant shallow-water forms belong to just those animal groups which are most characteristic of the deep-sea fauna. Third, the degree of tolerance of individual species is dramatically affected by other environmental variables such as temperature, salinity and pH, and those animals which are most tolerant of variations in these factors are also most tolerant of high pressures (Flügel in *Marine Ecology*, vol. 1 (3) (edit. by O. Kinne) 1407-1450 (1972)).

For many years the potentially more illuminating experiments on the longer term effects of high but sub-lethal pressures were virtually impossible because of the difficulties of controlling such factors as the accumulation of metabolites and the depletion of oxygen in the small volumes of water in which the animals were necessarily confined. Modern experimental chambers which can be flushed under pressure have largely overcome this obstacle, but almost all of the available data are still from relatively shallow-living animals, so that the fascinating question of how deep-sea forms cope with their high ambient pressures remained unanswered.

Work on these deeper forms is just beginning and some of the most interesting results have been obtained from studies of the effects of pressure on the metabolism of oceanic pelagic crustaceans, many of which undergo diurnal

vertical migrations through several hundreds of metres and therefore through several tens of atmospheres,

In the epipelagic forms tested, that is those which spend the night at, or close to, the surface, and migrate to depths of 200 or 300 m during the day, increased pressure within their normal range of experience has little or no effect on the respiratory rate. Low temperature, on the other hand, again within the normal environmental range, has a marked depressant effect on respiration, suggesting that by slowing their metabolism at their deep, cold, daytime levels these animals save energy by means of their diurnal migrations, despite the effort of swimming up and down (Teal and Carey, *Deep Sea Res.* 14, 725-733; 1967).

In contrast, the mesopelagic migrants, spending the daytime at depths down to perhaps 1,000 m and rising to within 200 m or so of the surface at night, show an increased respiratory rate not only with increased temperature but also with increased pressure. Here the suggestion is that by a cunning balance of the two effects these animals manage to maintain a fairly constant level of metabolic activity throughout their migratory cycle, a necessity, it is argued, for predators who must be prepared to exploit a limited food supply wherever and whenever the opportunity presents itself (Teal, *Am. Zool.*, 11, 571-576; 1971).

But these mesopelagic animals, despite their diurnal plunges, are nevertheless relatively shallow forms considering the total depth range of the oceans as a whole, and pressures not greatly beyond their normal range elicit general behavioural responses similar to those of their very shallow inshore relatives. Moderate pressure increases, usually less than about 200 atm., cause an increase in activity tending to become convulsive at the upper end of the pressure range. Activity is suppressed at slightly higher pressures, though the animals may recover upon decompression, but further compression, often around 400-500 atmospheres, causes tetany and even quite short exposures are often lethal.

Hardly any deeper-living species have been used in these kinds of experiments, but there is some evidence of an increased pressure tolerance, as one might expect, in these forms. The bathypelagic ostracod *Gigantocypris mulleri*, for example, with a normal depth range extending to 1,500 m, shows no change in its activity level at pressures up to 200 atm. and a somewhat greater tolerance of exposure to 500 atm. than most shallower animals (MacDonald, Gilchrist, and Teal, *J. mar. biol. Ass. U.K.*, 52, 213-223; 1972).

In the latest contribution to the high

pressure literature (*Deep Sea Res.*, 22, 131-144; 1975) MacDonald and Teal have confirmed and extended some parts of these earlier results, but have demonstrated further complications in the general pressure story. Another bathypelagic species, this time the amphipod *Lanceola sayana*, showed broadly similar pressure responses to those of *Gigantocypris* whereas rapid compression caused convulsions in the mesopelagic decapods *Systellaspis debilis* and *Acantheephyra purpurea*, and immobilisation at 200 atm. or even less. Although these mesopelagic species had shown a definite enhancement of respiratory rate with increased pressure in the earlier work, no such relationship was found in the new longer term respiration experiments. The oxygen consumption rate was, however, increased by disturbance of any kind, including changing the water in the experimental chamber, emphasising the difficulty of interpreting present experimental results in terms of the animals' performance in the natural environment.

Pressure is clearly an important environmental variable in the sea; relatively shallow-water animals seem to make use of it in various ways while deeper living ones appear to avoid its consequences. There are many potentially fruitful lines of future research, but one of high priority must surely be to study the physiology of deep-sea animals collected and maintained at their usual environmental pressures instead of being committed, as now, to the rigours of a conventional net. It has been fashionable in recent years to blame the high temperatures of the near surface layers for the virtual impossibility of bringing up living animals from more than about 1,500 m. But considering the dramatic effects of high pressures on shallow-living organisms it is at least possible that exposing a deep-sea creature to ambient pressures tens or even hundreds of atmospheres less than its normal one doesn't do it much good, quite apart from the battering it must get in the net! There are already indications that some seemingly dead benthic animals may recover if they are quickly recompressed (Menzies, George and Paul, *Int. Rev. ges. Hydrobiol.*, 59, (2), 187-197; 1974); under constant pressure they might not have 'died' in the first place.

The recovery of benthic animals under pressure presents special problems, but the technology to do so with planktonic forms has apparently been available for some time (MacDonald and Gilchrist, in *Barobiology and the experimental biology of the deep sea* (edit. by R. W. Brauer), 394-407 (1972)). Its use is long overdue and many biologists await the outcome with interest; the pressure is surely on!

review article

EPIC as a window on the forces of nature

Geoffrey Manning*

A proposal has been made to the Science Research Council for the construction of a new high energy physics accelerator called EPIC: electron positron intersecting complex. Because of recent experimental discoveries which have challenged earlier theoretical ideas, there is a general expectation that a major advance in our understanding of the basic forces of nature is imminent. EPIC, if constructed, will open up a new energy regime that will provide vital information to guide this advance.

ALL that is known about the laws of physics is based on observations over a range of distances from 10^{-13} to 10^{29} cm and for times between 10^{-24} and 10^{18} s. All of these observations—the whole of mankind's knowledge of the physical universe—can be explained in terms of the four basic interactions, gravitation, electromagnetic, weak and strong (Table 1). Gravitation is the weakest of the forces, its strength is 10^{-38} that of the strong interaction, we have an adequate theoretical understanding of it and it will not be discussed further here. The strong interaction, which is responsible for the binding of neutrons and protons within the atomic nucleus, has a range of $\sim 10^{-13}$ cm, is mediated by the exchange of particles of non-zero mass such as π mesons or K mesons. It is the strongest of the forces and we have no deep theoretical understanding of its behaviour. The electromagnetic interaction is responsible for the force between charged particles, its strength is $1/137$ that of the strong force, its range is infinite and it is mediated by the exchange of photons—the zero mass quanta of the electromagnetic field. The remaining force—the weak interaction—is responsible for the decay of many, otherwise stable, particles—for example, for the decay of the neutron into a photon, electron and neutrino ($n \rightarrow p + e^- + \nu$). The neutrino is a neutral particle of zero mass that has no strong interaction and only feels the weak interaction which is 10^{-5} that of the strong interaction. Experimentally the weak interaction seems to have zero range and no particle has been discovered that could mediate the interaction if its range is non-zero.

Our current understanding

Our present understanding of the three interactions relevant to elementary particle physics is very varied. The theory of the electromagnetic interaction—quantum electrodynamics (QED)—is well understood. It is possible to calculate quantities with great precision and as yet no discrepancy with experimental observation is known even though checks have been made to the level of about 1 part in 10^6 in the case of the magnetic moment of the electron and the muon¹. These high precision tests are sensitive to the detailed nature of the interaction at distances of $\gtrsim 10^{-14}$ cm—they test the perturbation expansion to high order. High energy, scattering experiments are necessary to test the theory at even smaller distances. The reaction $e^+ + e^- \rightarrow e^+ + e^-$ is dominated by the exchange of a single virtual photon and higher order terms—exchange of two

photons for example—contribute only at the 1% level. The reaction has been studied² at 5 GeV in the centre of mass confirming QED to distances $\sim 10^{-15}$ cm. Tests to even shorter distances require higher energies.

The apparently zero range of the weak interaction is assumed by the Fermi theory, which represents it as a point interaction and is very successful in correlating a large body of data. It predicts that the cross section for the weak interaction should increase as the square of the centre-of-mass energy, resulting in an infinite cross section as the energy tends to infinity. This cannot be and the theory is clearly wrong. We must give up the assumption of a point interaction and thus must have a particle to carry the force over the non-zero range. Experimental searches for this “intermediate vector boson” have been unsuccessful and its mass is probably greater than 20 GeV. Figure 1 shows the cross section for neutrinos on protons as a function of neutrino energy and indicates the predicted cross section if an intermediate boson is assumed to exist. The assumed existence of a charged intermediate vector boson cures the problem of the increase in cross section with energy but the higher order corrections corresponding to the exchange of more than one such boson are infinite: the theory is divergent.

The past few years have brought a major theoretical advance. Several ideas have been developed which assume a fundamental

Table 1 Properties of the four basic forces in nature

Force	Form of interaction	Relative strength	Range	Mediator
Gravity	Acts between any particles having mass	$\sim 10^{-38}$	Infinite	Graviton (zero mass)
Electromagnetic	Acts between charged particles	$\sim 10^{-2}$	Infinite	Photon (zero mass)
Weak	Responsible for decay of otherwise stable particles, for example, $n \rightarrow p + e^- + \nu$	$\sim 10^{-5}$	Seems to be zero $< 10^{-15}$ cm	No suitable particle seen experimentally
Strong	Binding force between nucleons in nuclei	1	$\sim 10^{-13}$ cm	Many particles known, for example, π meson, K meson

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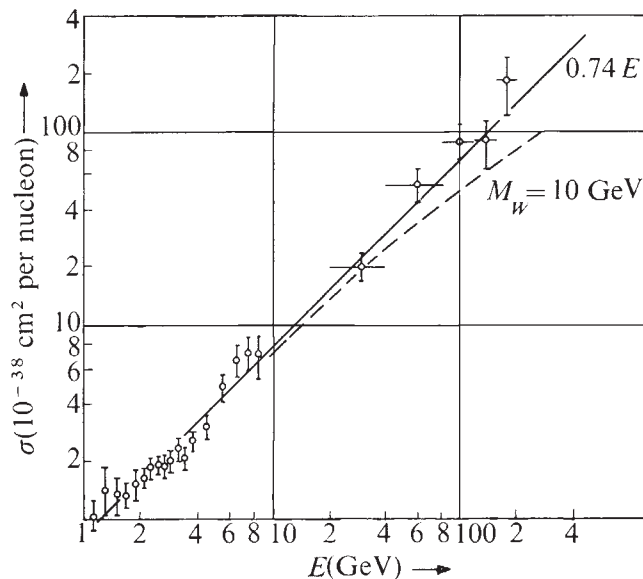


Fig. 1 Experimental results for the cross section of neutrinos on nucleons. The results are consistent with the cross section increasing linearly with energy—that is with a point interaction. The dashed line shows the cross section predicted on the assumption that the weak interaction is mediated by an “intermediate vector boson” of mass 10 GeV.

unifying connection between the weak and electromagnetic interactions. Many of these theories require the existence of a neutral intermediate vector boson as well as the charged bosons. Experiments at CERN³, later confirmed at FNAL⁴, have shown conclusive evidence for the existence of neutral current contributions to neutrino interactions compatible with the predictions of the unified theories; but direct evidence for the existence of neutral or charged intermediate vector bosons has yet to be found and is unlikely until experiments can be made at higher energies.

The unification of two of the basic forces of nature is analogous to the great advance made by Clerk Maxwell in the nineteenth century when he combined the then separate theories of electric and magnetic phenomena within a single electromagnetic framework. This was the basis of our modern theory of electromagnetism and its ramifications were immense. The new ideas for unifying weak and electromagnetic interactions may be extendable to include the strong interaction and even gravitation—if so it is difficult to exaggerate its significance.

Particles that interact through the strong interaction are called hadrons. They can be divided into two main groups—baryons and mesons. Baryons are similar to the proton but all others are unstable and decay resulting eventually in a single proton in the final state, excess energy being carried off by the emission of electrons, neutrinos or gamma rays. Often the break-up involves a complicated chain of decays. This conservation of protons is recognised by allocating all baryons a quantum number, the baryonic charge, $B = 1$. As far as we know this baryon quantum number is completely conserved in all reactions. There is a corresponding set of antibaryons ($B = -1$) each decaying and leaving a stable antiproton in the final state.

Mesons are strongly interacting particles with $B = 0$. They are all unstable and decay ultimately into electrons, neutrinos or gamma rays. If they are massive enough they can decay into a baryon-antibaryon pair as this conserves baryon number.

Throughout the past 10–20 years a great body of data on strong interactions has accumulated. The number of hadrons known has increased dramatically and is now several hundred. This glut was difficult to reconcile with the concept that they

Table 2 Properties of quarks and antiquarks in three-quark model

		Charge	Baryon number (B)	Strangeness (S)	Spin	Parity
Quarks	u	$\frac{2}{3}$	$\frac{1}{3}$	0	$\frac{1}{2}$	+
	d	$-\frac{1}{3}$	$\frac{1}{3}$	0	$\frac{1}{2}$	+
	s	$-\frac{1}{3}$	$\frac{1}{3}$	-1	$\frac{1}{2}$	+
Antiquarks	$\bar{u}$	$-\frac{2}{3}$	$-\frac{1}{3}$	0	$\frac{1}{2}$	-
	$\bar{d}$	$\frac{1}{3}$	$-\frac{1}{3}$	0	$\frac{1}{2}$	-
	$\bar{s}$	$\frac{1}{3}$	$-\frac{1}{3}$	1	$\frac{1}{2}$	-

were elementary and a major advance was made when all known hadrons were classified in terms of six basic building blocks: three quarks and their corresponding antiquarks (Table 2). Each of these quarks is assumed to have $B = \frac{1}{3}$ and the antiquarks $B = -\frac{1}{3}$. Baryons are built of three quarks giving $B = 1$; antibaryons from three antiquarks; and mesons from one quark and one antiquark.

To account for other conserved properties discovered by experiments in strong interaction physics, quarks are given other quantum numbers including “strangeness”. Strangeness is additive as electric charge and baryonic charge are additive and is conserved in the strong interaction although it is not conserved by the weak interaction.

The quark model not only classifies all known states but also gives approximate values for the masses, the decay rates, the magnetic moments, relationships between cross sections and so on. Its considerable success, however, is limited by several weaknesses, some theoretical but one major one of which is that experimental attempts to create and observe free quarks have failed. This could mean that quarks are very massive or that they are just a mathematical abstraction.

Further and more direct evidence for a substructure to the proton has been provided by experiments involving electron scattering. The behaviour of inelastic electron scattering from protons can be simply explained by assuming that a point-like electron is scattered from a point-like constituent charged part of the proton by the electromagnetic interaction. Similar and complementary evidence is obtained from neutrino scattering involving the weak interaction. These experiments not only directly demonstrate the existence of a substructure but they also measure the properties of the constituent parts. The results are consistent with the assumed properties of the quarks.

The recent discovery⁵⁻⁷ of two new particles of mass 3.1 and 3.7 GeV with widths a factor of one thousand less than that normally expected, and a further state at 4.2 GeV (ref. 8) with a more normal width, has raised the question of yet another theoretical proposition: a fourth quark with a new quantum number, charm⁹. Previously discovered particles are constituted of the three quarks listed in Table 2 and the new states contain quarks with charm +1 and/or antiquarks with charm = -1.

Table 3 Properties of quarks and antiquarks in four-quark model

		Charge	Baryon number (B)	Strangeness (S)	Spin	Parity	Charm
Quarks	u	$\frac{2}{3}$	$\frac{1}{3}$	0	$\frac{1}{2}$	+	0
	d	$-\frac{1}{3}$	$\frac{1}{3}$	0	$\frac{1}{2}$	+	0
	s	$-\frac{1}{3}$	$\frac{1}{3}$	1	$\frac{1}{2}$	+	0
	c	$\frac{2}{3}$	$\frac{1}{3}$	0	$\frac{1}{2}$	+	1
Antiquarks	$\bar{u}$	$-\frac{2}{3}$	$-\frac{1}{3}$	0	$\frac{1}{2}$	-	0
	$\bar{d}$	$\frac{1}{3}$	$-\frac{1}{3}$	0	$\frac{1}{2}$	-	0
	$\bar{s}$	$\frac{1}{3}$	$-\frac{1}{3}$	1	$\frac{1}{2}$	-	0
	$\bar{c}$	$-\frac{2}{3}$	$-\frac{1}{3}$	0	$\frac{1}{2}$	-	-1

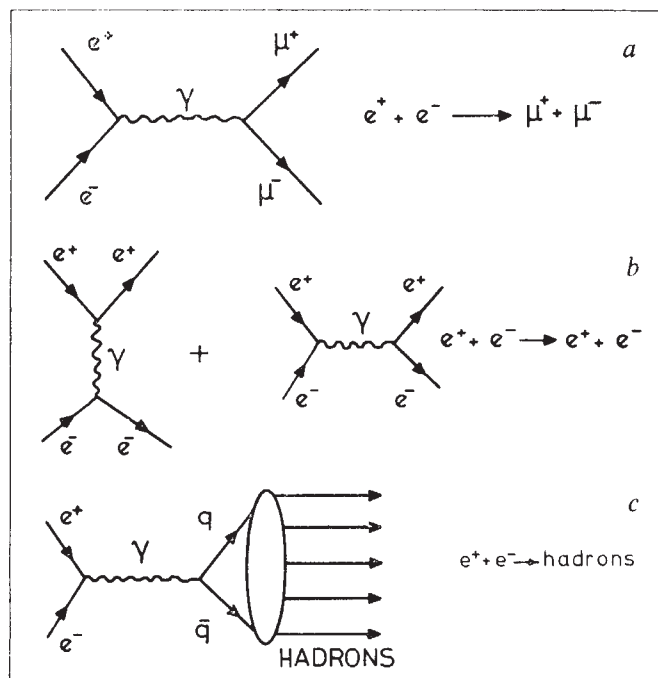


Fig. 2 Diagrammatic representation of the one photon contribution to the reactions: *a*, $e^+ + e^- \rightarrow \mu^+ + \mu^-$; *b*, $e^+ + e^- \rightarrow e^+ + e^-$ and *c*, $e^+ + e^- \rightarrow \text{hadrons}$ through production of a quark-antiquark pair.

Table 3 lists the assumed properties of the four quarks and four antiquarks. The existence of a fourth quark is also required to explain the absence of neutral current effects in some regions of the weak interaction (in neutral K^0 decays.) The new particles are still under investigation and theoretical problems remain

but there is a strong feeling that high energy physics is on the point of a breakthrough.

Why EPIC?

EPIC¹⁰ is an accelerator in which high energy (14 GeV) positrons are collided head-on with electrons of the same energy. The whole energy of the electron and positron is available in the centre-of-mass system. In contrast if a positron hits a stationary electron essentially all of the energy is used in producing motion of the centre of mass and to achieve 28 GeV centre-of-mass energy in this way would require a positron energy of 800,000 GeV.

Why is it necessary or desirable to study e^+e^- reactions? Are not pp studies adequate? e^+e^- reactions are predominately electromagnetic. The annihilation of an initial e^+e^- state to give any final state is dominated by the exchange of a single virtual photon as indicated in Fig. 2*a* for the reaction $e^+ + e^- \rightarrow \mu^+ + \mu^-$; in Fig. 2*b* for the two contributions to $e^+ + e^- \rightarrow e^+ + e^-$, and in Fig. 2*c* for the reaction $e^+ + e^- \rightarrow \text{hadrons}$ on the assumption that production of hadrons is a result of the production of a quark-antiquark pair. In all cases the contribution from the exchange of two photons is of the order of 1%. The domination of the single photon contribution means that the final state has a well defined quantum state with a given spin, parity and charge quantum number ($J^{PC} = 1^{--}$). In contrast the pp reaction with a centre-of-mass energy of 28 GeV would have contributions from a hundred different values of J . The uniqueness of the final state brings strong constraints and rather precise predictions on what is expected. Experiments can therefore check the basic assumption that QED works to distances of $\sim 10^{-16}$ cm: that is, to volumes of 10^{-9} of the proton volume. The energy density reached in these reactions is $\sim 10^{49}$ GeV cm⁻³—orders of magnitude larger than that found in astrophysics—and we have no right to assume that laws tested elsewhere will of necessity still apply in this new energy regime.

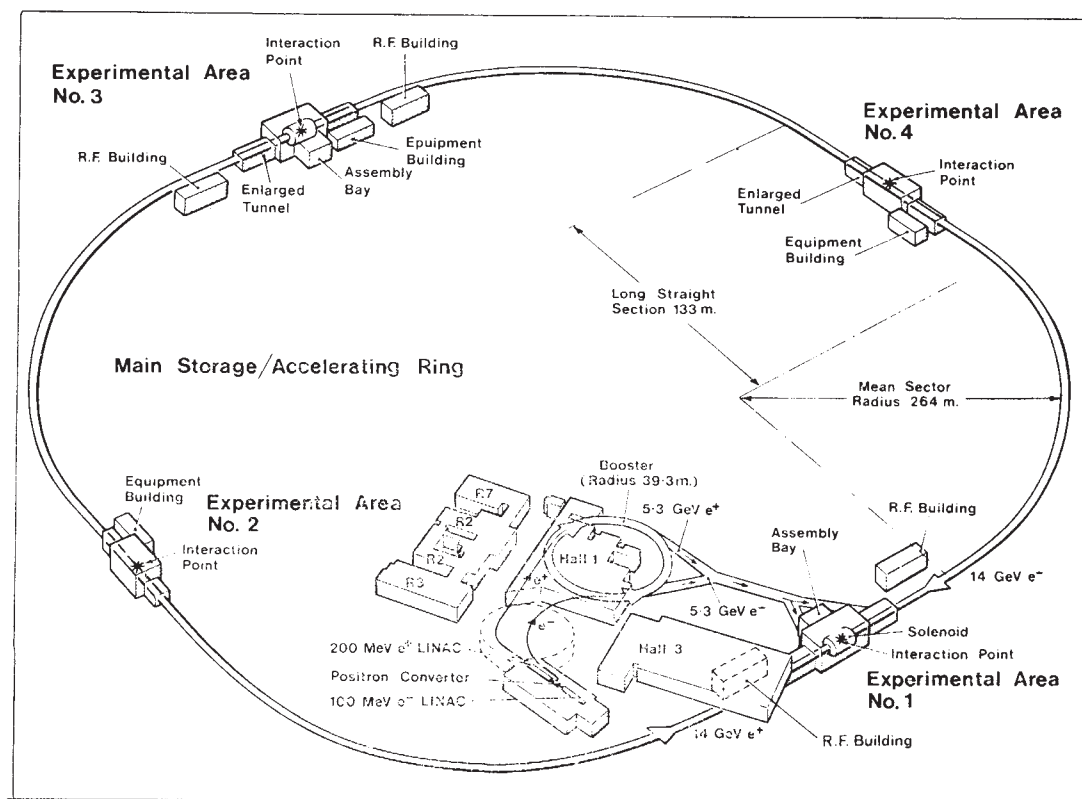


Fig. 3 Diagram of EPIC showing major components. The booster, one of the LINACS and the buildings shown in the booster area already exist.

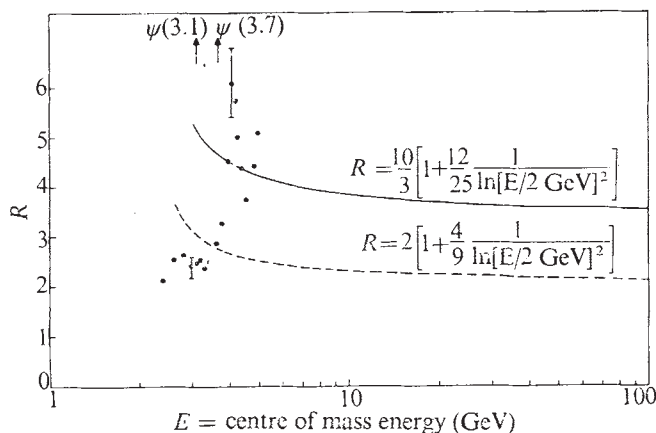


Fig. 4 Experimental values of

$$R = \frac{\text{cross section for } e^+e^- \rightarrow \text{hadrons}}{\text{cross section for } e^+e^- \rightarrow \mu^+\mu^-}$$

and the theoretical predictions for two different quark models. The lower energy region includes resonances. At EPIC energies one should be close to the asymptotic values and should be able to 'determine' the form of the substructure of hadrons.

At EPIC energies one can expect to see contributions from other interactions—from the weak interaction caused by the exchange of neutral vector bosons—from the strong interaction testing the properties of the constituent parts and probing their structure to distances $\sim 10^{-16}$ cm. The existence of a good theory for the electromagnetic reaction provides a fine probe for investigating the unknown: it is analogous to probing the structure of a watch using X rays rather than by throwing two watches at each other and studying the resulting parts, which is closer to the situation when one studies pp reactions.

Achieving electron-positron collisions

If a beam of electrons is fired at a beam of positrons the majority of the particles miss each other because the cross sections are very small ($\sim 10^{-34}$ cm²) at EPIC energies. To compensate for this the particles are contained within a toroidal vacuum chamber by means of guiding magnetic fields so that they can be reused. The electrons rotate one way and the positrons counter-rotate within the same vacuum chamber. Bending the particles around to reuse them results in energy loss by synchrotron radiation (emission of short wavelength light) and on average each electron and positron loses 20 MeV per revolution. 3×10^{12} particles are stored in EPIC and they make 1.4×10^5 circuits per s resulting in $\sim 10^{19}$ MeV lost per s or 1.4 MW. This loss has to be compensated by accelerating cavities operating at about 400 MHz—about 3–4 MW of power are provided to the cavities to replace the 1.4 MW lost by the beams. The energy lost per turn by an electron is $\propto E^4/R$ where E is the energy of the electron and R is the radius of the machine. It is this fact that limits the energy that can be used and forces the radius of EPIC to such a large value (Fig. 3). The circumference of EPIC is 2.2 km.

The radio frequency acceleration bunches the electrons and positrons. In EPIC there are two bunches of electrons diametrically spaced and two similarly spaced bunches of positrons rotating in the opposite direction. The particles are phased so that they cross in the centre of the four long straight sections (Fig. 3). At these four interaction points the bunches of particles are strongly focused to increase the interaction rate—the vertical height at this point is ~ 0.015 cm, the horizontal width is 0.06 cm and the length ~ 10 cm. It is not possible to

focus the bunches even more strongly because of beam-beam interactions which are dependent on current density. The interaction rate is determined by the properties of storage ring and the cross section to be studied, so that

$$\text{Interaction rate} = \sigma L$$

L is the luminosity of the storage ring and the design value for EPIC at 28 GeV centre-of-mass energy is 5×10^{31} cm⁻² s⁻¹. For a typical cross section of 10^{-34} cm² this gives a count rate of 18 h⁻¹ at each intersection region. This count rate is not large but is quite adequate to do the physics envisaged. Some cross sections are much larger and have higher event rates.

Electrons and positrons have to be injected into the EPIC main ring and the plan is to use the 5 GeV electron accelerator NINA and associated equipment. NINA will be moved from the Daresbury Laboratory to the Rutherford Laboratory when the physics programme at Daresbury ends in 1977. Buildings and ancillary plant currently used for the 7 GeV proton accelerator NIMROD at the Rutherford Laboratory will also be used wherever possible. It is planned to stop operation of NIMROD in 1979 and EPIC should be ready to start commissioning its first physics experiments in 1980.

The accumulation of the required 8×10^{11} positrons in each of the two bunches in EPIC represents the principal problem in the injection process. Electrons are accelerated to 0.1 GeV in a linear accelerator and are focused on to a metal target resulting in the production of electron-positron pairs. The positrons are collected and accelerated to 0.2 GeV in a second linear accelerator. These positrons are further accelerated in NINA to an energy of 5.3 GeV before they are injected into EPIC. About ten thousand such cycles are needed to accumulate the required number of positrons and this takes about three minutes. The filling of the electron bunches in EPIC is easier as the positron conversion efficiency is about 10^{-3} and thus for electrons very few NINA acceleration cycles are required. The total filling time is expected to be less than four minutes.

The two bunches of electrons and two bunches of positrons in EPIC are accelerated from 5.3 GeV to 14 GeV in about one minute and the full energy beams can then be stored for several hours while interactions take place at the four interaction regions. The beams are gradually attenuated as a result of the beam-beam interactions and the scattering on the residual gas even though the vacuum is kept at 10^{-9} mmHg.

If more radio frequency cavities are added it is possible to increase the energy of EPIC to about 20 GeV per beam, 40 GeV centre-of-mass energy, but at a reduced luminosity.

What experiments can one do with EPIC?

The potential contribution of EPIC in high energy physics is fourfold. First, it may help to answer some outstanding questions on hadron substructure. Experimental results on e^+e^- annihilation into hadrons made at the SPEAR storage ring at the Stanford Linear Accelerator Laboratory, USA, and at other lower energy storage rings show that the cross section for the production of hadrons is much larger than would originally have been expected^{2,8}. The large cross sections are direct evidence that the hadron production occurs through some particles of very small dimension; the current assumption is that it is through production of a quark-antiquark pair (see Fig. 2c). This model predicts that the ratio

$$R = \frac{\text{cross section for } e^+e^- \rightarrow \text{hadrons}}{\text{cross section for } e^+e^- \rightarrow \mu^+\mu^-} = \sum_i q_i^2$$

where q_i is the electronic charge of the quark and the summation is overall possible quarks. Thus for the three quark model one predicts $R = (\frac{1}{3})^2 + (\frac{1}{3})^2 + (\frac{2}{3})^2 = \frac{5}{3}$ and for the four quark model $R = (\frac{1}{3})^2 + (\frac{1}{3})^2 + (\frac{2}{3})^2 + (\frac{2}{3})^2 = \frac{10}{9}$.

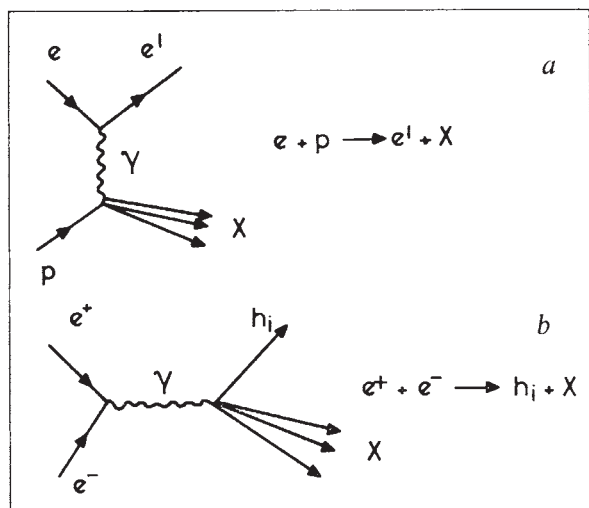


Fig. 5 *a*, Reaction $e + p \rightarrow e' + \text{anything}$. It can be thought of as a massive virtual photon probing the structure of the proton. As the probed particle is the target only neutrons and protons can be studied this way. *b*, Represents the reaction $e^+ + e^- \rightarrow h_i + \text{anything}$. Similarly this may be thought of as a massive virtual photon probing the structure of the hadron h_i . Any hadron that is produced can be studied in this way including unstable particles such as pions, kaons.

There are more complicated quark models predicting that three varieties of each quark should exist¹¹ with an additional label: colour. For such a model without charm $R = 2$, with both charm and colour $R = 10/3$. More complicated models¹² exist that predict even larger values of R .

The prediction is that at high energies R should be independent of energy and that its value is dependent on the assumed building blocks for the hadrons. At lower energies resonances will be seen corresponding to the production of particular hadron states: the new particles at 3.1, 3.7 and 4.2 GeV are examples of this. There are model dependent predictions on the approach to the asymptotic value of R and Fig. 4 shows these predictions¹³ and the existing experimental results. EPIC will be able to reach the asymptotic region and will determine which, if any, of the quark models is correct.

More information can be obtained by studying the details of the particle production. As mentioned above, deep inelastic electron scattering, $e + p \rightarrow e' + \text{anything}$, has been used to probe the structure of the proton and Fig. 5*a* shows the reaction diagrammatically: the virtual photon probes the structure of the proton. Figure 5*b* shows the reaction $e^+ + e^- \rightarrow h_i + \text{anything}$ where h_i can be any hadron (for example a proton, a pion, a kaon and so on). The reactions are related and one can think of reaction 5*b* as probing the structure of the hadron h_i . This process has the advantage of being able to probe unstable as well as stable hadrons whereas inelastic electron scattering requires a stable target and is thus limited to protons or neutrons. Measurements will include single particle production to test scaling; determination of particle ratios as a function of particle momentum; determination of particle correlations; measurement of specific exclusive channels and so on. EPIC opens a new range of energies for such studies and allows a much deeper investigation of hadron substructure than is currently possible.

The second issue which EPIC might help to resolve is the distance over which quantum electrodynamic equations are valid. The experimental² values of $\sigma(e^+ + e^- \rightarrow \text{hadrons})$ is constant as $\sim 2 \times 10^{-32} \text{ cm}^2$ for centre-of-mass energies from 3 to 5 GeV. Should this value continue to 28 GeV strong interaction corrections to the photon propagator would produce

measurable effects and a breakdown of the normal QED perturbation expansion would be evident¹⁴.

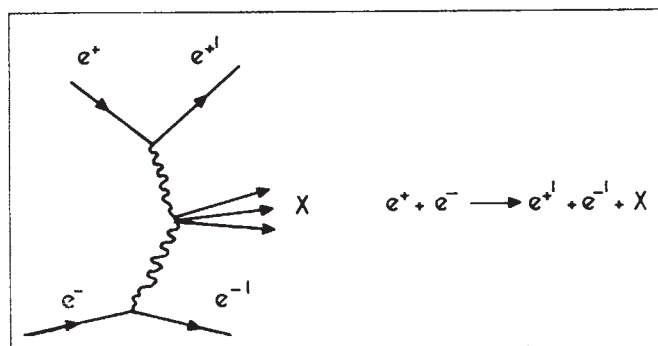
There are three main reactions that can be used to test QED at EPIC; $e^+ + e^- \rightarrow e^+ + e^-$; $e^+ + e^- \rightarrow \mu^+ + \mu^-$ and $e^+ + e^- \rightarrow 2\gamma$. All can be studied experimentally and QED makes detailed predictions about the cross sections and angular distributions expected. Such measurements will test the validity of QED to distances $\sim 10^{-16} \text{ cm}$ —a factor of ten further than currently possible. Different experimental conditions can be chosen to check photon and lepton propagators over a wide range of momentum transfers.

Third, EPIC energies could clarify outstanding problems with the weak force. The reaction $e^+ + e^- \rightarrow \mu^+ + \mu^-$ is dominated by the exchange of a single photon (Fig. 2*a*). Contributions are also possible from the weak interaction through the exchange of a neutral intermediate boson. At EPIC energies this effect is about 3% but there is a further contribution from interference between the weak interaction and the electromagnetic interaction that is $\sim \sqrt{3\%} \approx 17\%$. The exact level of the effect is dependent on the form of the neutral current contribution to the weak interaction. The ratio of the weak interaction contribution to $e^+ + e^- \rightarrow \mu^+ + \mu^-$ to that from the electromagnetic interaction increases as the fourth power of the centre-of-mass energy and thus it is important to run EPIC at as high an energy as possible. The weak interaction modifies the QED predictions by changing the rate for the reaction, changing the angular distribution and by producing polarisation effects.

Such effects can be measured even if the mass of the neutral mediator of the weak interaction is too large for the particle to be directly produced. Different unified theories for the weak and electromagnetic interactions make specific predictions. For example the Weinberg-Salam model predicts a particle Z_0 of mass greater than 79 GeV, but its existence produces detectable effects in the $e^+ + e^- \rightarrow \mu^+ + \mu^-$ reaction at EPIC energies. Experiments will thus be able to test these predictions, decide which model is correct and possibly determine the mass of the neutral mediator.

So far I have concentrated on the domination of one photon exchange in annihilation of e^+e^- . The fourth area in which EPIC might make an important contribution is that of non-annihilation reactions in which two-photon contributions dominate. Such reactions are $e^+ + e^- \rightarrow e^+ + e^- + X$ where X stands for any final state. Figure 6 represents this diagrammatically. The reaction can be thought of as the collision of two virtual photons to form the final state X . The cross section for such reactions can be large and the reactions can be identified by detecting and measuring the e^+ and e^- in the final state. A whole class of interesting physics can be studied in this way that cannot be tackled by other means. At available storage rings the cross sections are small but at EPIC energies

Fig. 6 Representation of the reaction $e^+ + e^- \rightarrow e^+ + e^- + X$, where X represents any final state. Different energies and directions of the final state inelastically scattered electron and positron (e^+ and e^-) and final states X can be selected to study a wide range of important physics.



they are predicted to exceed the annihilation channels by more than a factor of 100.

One of the most interesting studies is to choose the energies and direction of the e^+ and e^- in the final state so that one of the virtual photons is almost real (a mass less than the mass of the pion) and the second virtual photon is massive. This corresponds to a virtual photon probing the structure of the photon itself. Does the photon have constituent parts?

Feasibility of EPIC

The UK high energy physics community has put a great deal of effort into a feasibility study of EPIC^{10,15}. The cost of the project is estimated to be £25.7 million. In spite of projected decreases in the funds for Nuclear Physics, the Nuclear Physics Board of the Science Research Council has fitted the project within its future plans by planning to close the existing two domestic high energy physics accelerators NINA and NIMROD in 1977 and 1979 respectively. The operation and utilisation costs for these accelerators is diverted into construction money for EPIC and ultimately into operation and utilisation money for the single facility.

The Science Research Council has accepted that there is a strong scientific case for the construction of EPIC and has included it within its future plans provided that some international contribution can be found towards its construction and operation.

Within Europe there is a second proposal for a high energy e^+e^- colliding beam machine—namely PETRA¹⁶, which is proposed by the DESY Laboratory in Germany. European high energy physicists as represented by ECFA (European Committee for Future Accelerators) have recommended that one or other of the two projects should be supported and constructed. It is generally accepted that whichever is built international participation in the utilisation is certain.

British physicists argue that EPIC is to be preferred as DESY should concentrate on developing and using their lower energy storage ring DORIS that is just starting operation. A further argument in favour of EPIC is that it has been designed so that a magnet ring for protons can be added above the electron-positron ring and within the same tunnel. This addition will allow for the study of ep reactions. This is an option for the future that is not available in the PETRA design.

British high energy physicists argue strongly that an opportunity exists for a unique and exciting project to be constructed in the UK. The physics that can be studied is at the forefront of our understanding. It is in an area that we have the necessary expertise within the universities and the Science Research Council to exploit. German physicists argue equally strongly for the construction of PETRA in Germany. The choice is difficult, but it is important that a decision should be made and that a European e^+e^- project should be started as soon as possible.

¹ de Rafael, E., *Introduction to quantum electrodynamics*, Proc. Ecole Internationale de la Physique des Particules Elementaires (University of Strasbourg, Strasbourg, 1972).

² Richter, B., Proc. XVII Int. Conf. High Energy Physics London, 4, 37 (Rutherford Laboratory, 1974).

³ Hasert, F. J., et al., Nucl. Phys. B, 73, 1 (1974).

⁴ Aubert, B., et al., Phys. Rev. Lett., 32, 1454 (1974).

⁵ Augustin, J. E., et al., Phys. Rev. Lett., 33, 1406 (1974).

⁶ Aubert, J. J., et al., Phys. Rev. Lett., 33, 1404 (1974).

⁷ Abrams, G. S., et al., Phys. Rev. Lett., 33, 1453 (1974).

⁸ Augustin, J. E., et al., Phys. Rev. Lett., 34, 764 (1975).

⁹ Glashow, S. L., Iliopoulos, J., and Maiani, L., Phys. Rev. D, 2, 1285 (1970).

¹⁰ Rutherford Laboratory Report RL-74-100 (1974).

¹¹ Greenberg, O. W., Phys. Rev. Lett., 13, 598 (1964).

¹² Nambu, Y., and Han, M. Y., Phys. Rev., D, 10, 674 (1974).

¹³ Gaillard, M. K., Lee, B. W., and Rosner, L., FNAL-PUB-74/86—THY (1974).

¹⁴ Gatto, R., Phys. Lett. 51B, 371 (1974).

¹⁵ Rutherford Laboratory Report RL-74-124 (1974).

¹⁶ A proposal for extending the storage ring facilities at DESY to higher energies, DESY Report (November 1974).

articles

Methyl group as a probe of chirality in Raman optical activity

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Raman circular intensity differentials observed in methyl asymmetric deformations and methyl torsions indicate that the methyl group could function as a powerful new probe of chirality. Torsional Raman optical activity could lead to new knowledge of barriers to internal rotation in molecules and aid the assignment of torsional modes.

VIBRATIONAL optical activity can be studied through a difference in the intensity of Raman scattering in right and left circularly polarised incident light (see refs 1 to 5). The effect originates in interference between light waves scattered through the electronic polarisability and the electronic optical activity of the molecule. An appropriate experimental quantity is a normalised

circular intensity differential (CID),

$$\Delta_{\alpha} = (I_{\alpha}^R - I_{\alpha}^L) / (I_{\alpha}^R + I_{\alpha}^L)$$

where I_{α}^R and I_{α}^L are the scattered intensities with α polarisation in right and left circularly polarised incident light. (I_z and I_x are linearly polarised parallel and perpendicular to the scattering plane and Δ_z and Δ_x are referred to as the depolarised and polarised CIDs.) Routine Raman CID spectrometers have been described elsewhere (see refs 4 and 5).

Raman CIDs have been observed in many molecules, but because of the complexity of the spectra and the considerable mixing of the normal vibrational coordinates in the regions where the best effects are usually found (0–1,500 cm^{-1}), it has been difficult to assign the Raman CIDs to particular normal

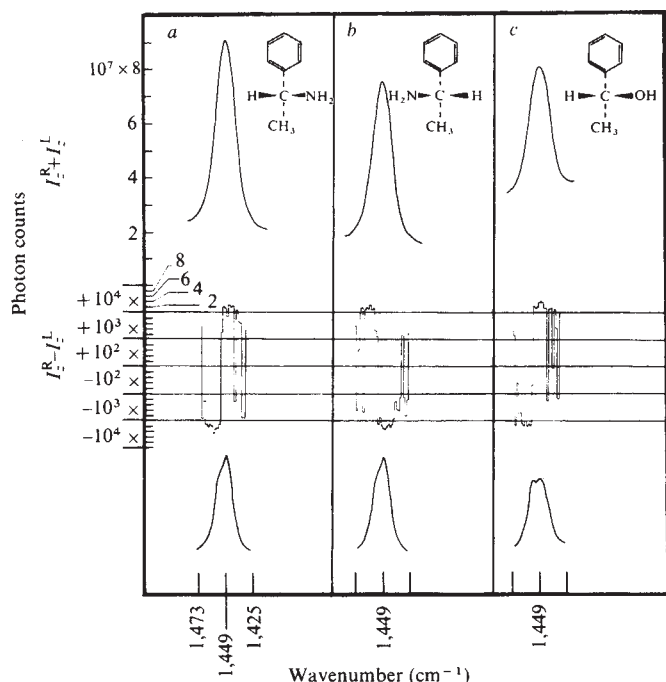


Fig. 1 The depolarised Raman circular intensity sum (top) and difference (centre) spectra, as recorded, of methyl asymmetric deformations in: *a*, (+)- α -phenylethylamine; *b*, (-)- α -phenylethylamine; *c*, (+)- α -phenylethanol. At the bottom is a sum spectrum at high resolution. Instrumental conditions: laser wavelength 4,880 Å, laser power 1 W, slit width 1,500 μ m for the top and centre spectra and 200 μ m for the bottom spectra. The absolute configurations are taken from ref. 10.

coordinates of vibration. In general, cyclic structures show larger effects than acyclic structures in which the existence of rotational conformers can reduce the chirality, although exceptions include molecules where four rigid groups are attached to an asymmetric carbon atom as in α -phenylethylamine.

Definite assignments of Raman optical activity to localised vibrations have now been made in the important case of the methyl group, and examples of Raman CIDs generated by methyl asymmetric deformations and methyl torsions are shown below. (For reasons discussed previously^{3,5}, only the depolarised CIDs are sampled. To estimate the normalised CIDs, the backgrounds should first be subtracted from the $I_z^R + I_z^L$ spectra.) By means of these effects, the methyl group could function as a specific and reliable probe of configuration, although there are insufficient data at present to attempt detailed correlations. Similar assignments will probably be made for vibrations in other groups, in particular deformations and torsions of $-\text{C}_6\text{H}_5$, $-\text{NH}_2$, $-\text{NH}_3^+$, $-\text{OH}$, $-\text{NO}_2$, and so on.

Methyl asymmetric deformations

Raman CID couplets are found in a depolarised band at about 1,450 cm^{-1} in α -phenylethylamine and α -phenylethanol and the spectra, as recorded, are shown in Fig. 1.

This effect in α -phenylethylamine was first noticed by Hug *et al.*⁴, who realised that it originated in the two degenerate asymmetric deformations of the methyl group and could function as a new probe of chirality. If the effective symmetry of the methyl group is reduced through bonding such that its threefold axis is lost, the degeneracy is lifted and a splitting can sometimes be seen in the infrared or Raman spectrum⁶. This splitting is visible in the Raman spectra of α -phenylethylamine and α -phenylethanol at high resolution (200 μ m), but not at the low resolution (1,500 μ m) used to scan the CIDs (Fig. 1).

The effective symmetry of a methyl group bonded to an asymmetric carbon atom is C_1 , so its electronic and vibrational transitions can support optical activity; here this takes the form of Raman CIDs of opposite signs in the two components of the methyl asymmetric deformation.

Similar effects should exist in molecules such as alanine, lactic acid and threonine in aqueous solution, but are below the present limits of detection; also in $-\text{NH}_3^+$ attached to asymmetric carbon atoms in all amino acids in acid aqueous solution. Examples of this effect are not as distinctive in methyl groups in molecules such as 2-amino-1-phenylpropane (amphetamine) and 2-octanol because of the existence of rotational conformers, nor in methyl groups attached to ring structures.

Methyl torsions

Raman CIDs are found in methyl torsion vibrations between about 200 and 300 cm^{-1} in most optically active molecules containing methyl groups. For example, (+)-3-methylcyclohexanone shows a broad weak depolarised Raman band at about 260 cm^{-1} with a large CID ($\Delta_z \sim -2.5 \times 10^{-3}$, Fig. 2*a*); this can be assigned to the methyl torsion which is known to occur between about 230–280 cm^{-1} in methylcyclohexanes⁷. The corresponding band in (+)-3-methylcyclopentanone may be that at about 290 cm^{-1} ($\Delta_z \sim -1.5 \times 10^{-3}$, Fig. 2*b*). Similar bands appear in (-) limonene ($\Delta_z \sim -1.5 \times 10^{-3}$, Fig. 2*c*) and (+) carvone ($\Delta_z \sim +1.8 \times 10^{-3}$, Fig. 2*d*), but now the interpretation is no longer unequivocal since both molecules possess two methyl groups. It is possible that the bands shown at about 250 cm^{-1} originate in the isopropenyl methyl group since the corresponding torsional barrier, and hence the torsional frequency, is expected to be higher than for the ring methyl group (methyl torsions in *ortho*-substituted toluenes occur around 170 cm^{-1} , ref. 8).

A dramatic example of torsional Raman optical activity is found in dimethyldibenz-1,3-cycloheptadiene-6-one (Fig. 2*e*), a bridged biphenyl in which two equivalent methyl groups twist in a highly chiral environment. Coupling between the two methyl torsions generates two Raman bands, associated with symmetric and asymmetric combinations of the local modes, centred at about 234 cm^{-1} . An enormous Raman CID ($\Delta_z \sim -7 \times 10^{-3}$) is found in the lower frequency band, but none in the higher frequency band.

Unfortunately torsional modes other than methyl are often difficult to assign unambiguously as the frequencies are very variable and can overlap with low frequency skeletal modes. On the other hand, Raman optical activity could be of considerable assistance in assigning torsional modes in optically active molecules. In fact the first reported observations³ of Raman

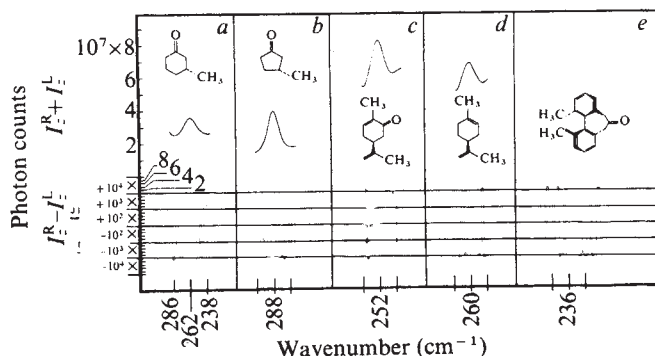


Fig. 2 The depolarised Raman circular intensity sum and difference spectra of methyl torsions in: *a*, (+)-3-methylcyclohexanone; *b*, (+)-3-methylcyclopentanone; *c*, (+)-carvone; *d*, (-)-limonene; *e*, a saturated solution of (+)-dimethyldibenz-1,3-cycloheptadiene-6-one in acetone. Instrumental conditions as for the top two spectra in Fig. 1, except that a laser wavelength of 5,145 Å was used in *e* to minimise fluorescence.

CID in α -phenylethylamine and α -phenylethanol between about 200 and 400 cm^{-1} probably involve $-\text{CH}_3$, $-\text{NH}_2$ and $-\text{OH}$ torsions.

Raman optical activity is determined by products such as^{1,5}

$$\begin{aligned} & \langle \nu^i | \alpha_{\alpha\beta} | \nu^f \rangle \langle \nu^f | G'_{\alpha\beta} | \nu^i \rangle \\ &= \sum_j \left(\frac{\partial \alpha_{\alpha\beta}}{\partial Q_j} \right)_e \left(\frac{\partial G'_{\alpha\beta}}{\partial Q_j} \right)_e |\langle \nu^f | Q_j | \nu^i \rangle|^2 \end{aligned}$$

where ν^i and ν^f are the initial and final vibrational states, Q_j is the j th normal vibrational coordinate, $\alpha_{\alpha\beta}$ is the electric dipole–electric dipole polarisability tensor, $G'_{\alpha\beta}$ is the electric dipole–magnetic dipole optical activity tensor and $A_{\alpha\beta\gamma}$ is the electric dipole–electric quadrupole optical activity tensor. So the Raman CID associated with the methyl torsion originates in the products of the changes in electronic polarisability and electronic optical activity, evaluated at the equilibrium nuclear configuration, as the methyl group twists about its threefold axis in the chiral environment from the rest of the molecule. For ‘static coupling’ of the methyl group with the chiral environment, a sextant symmetry rule is predicted².

Since torsional Raman optical activity arises from the chiral part of the electronic potential barrier hindering the motion, it could provide a novel approach to the study of internal rotation in molecules, and to conformational analysis generally. For example, in the chair form of 3-methylcyclohexanone with the methyl group occupying the equatorial position, most of the potential barrier hindering the methyl torsion arises from the two flanking methylene groups. The presence of the sp^2 hybridised atom in the ring results in an unsymmetrical chair so that the two methylenes flanking the methyl are slightly twisted relative to each other. In the (+) enantiomer the twist is left-

handed and is probably responsible for the negative methyl torsional Raman optical activity, although the theory is not sufficiently developed to predict with confidence the sign from the sense of twist. It is therefore possible that the negative methyl torsional optical activity in (+)-3-methylcyclopentanone arises because in this molecule too there is a left-handed twist in the two methylenes flanking the methyl. Although little is known about the conformations of five-membered rings, this could be consistent with the suggested requirements⁹ for the methyl to occupy the quasi-equatorial position in the envelope form of methylcyclopentane and for ring puckering at the carbon atoms away from the sp^2 hybridised atom in cyclopentanone.

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¹ Barron, L. D., and Buckingham, A. D., *Molec. Phys.*, **20**, 1111 (1971).

² Barron, L. D., *J. chem. Soc. A*, 2899 (1971).

³ Barron, L. D., Bogaard, M. P., and Buckingham, A. D., *J. Am. chem. Soc.*, **95**, 603 (1973).

⁴ Hug, W., Kint, S., Bailey, G. F., and Scherer, J. R., *J. Am. chem. Soc.* (in the press).

⁵ Barron, L. D., and Buckingham, A. D., *Ann. Rev. phys. Chem.*, (in the press).

⁶ Herzberg, G., *Infrared and Raman Spectra of Polyatomic Molecules*, 334 (Van Nostrand, Princeton, 1945).

⁷ Sushchinskii, M. M., *Raman Spectra of Molecules and Crystals*, 207 (Israel Programme for Scientific Translations, Jerusalem, 1972).

⁸ Finch, A., Gates, P. N., Radcliffe, K., Dickson, F. N., and Bentley, F. F., *Chemical Applications of Far Infrared Spectroscopy*, 88 (Academic Press, London, 1970).

⁹ Pitzer, K. S., and Donath, W. E., *J. Am. Chem. Soc.*, **81**, 3213 (1959).

¹⁰ Klyne, W., and Buckingham, J., *Atlas of Stereochemistry* (Chapman and Hall, London, 1974).

Stringent control of the transcriptional activities of ribosomal protein genes in *E. coli*

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The stringent control system regulates the expression of not only rRNA genes but also r-protein genes. The regulation of r-protein genes takes place by a mechanism which determines the amount of r-protein mRNA available for translation. Presumably this mechanism operates at the level of initiation of transcription at r-protein gene promoters.

In the bacterium *Escherichia coli*, the synthesis of ribosomal components is regulated^{1,2}. One aspect of this regulation which has been studied extensively is the stringent control of stable RNA synthesis; during amino acid (or charged tRNA) deprivation in stringent (*rel*⁺) strains stable RNA accumulation is strongly depressed, whereas in mutant relaxed (*rel*[−]) strains RNA accumulation continues unabated^{2–4}. In contrast to stable RNA, it has been thought that amino acid starvation does not affect the synthesis of mRNA to any great extent^{3–6}.

We have shown previously that the synthesis of ribosomal proteins (r-proteins) like rRNA is subject to the influence of the stringent control system⁷. We used isogenic *rel*⁺ and *rel*[−] strains carrying a temperature sensitive mutation in the valyl-tRNA synthetase gene (*ValS*); at growth temperatures above

32 °C the level of amino acylated valyl-tRNA becomes deficient and therefore the effect of charged tRNA deprivation on RNA and protein synthesis can be studied. It was observed that during partial inhibition of tRNA charging in these strains, the synthesis of individual r-proteins remained in balance and closely coupled to the accumulation of stable RNA⁷. That is, in the *rel*⁺ strain, the synthesis of stable RNA (that is, accumulation) and r-protein was more strongly inhibited than total protein synthesis and the differential rate of r-protein synthesis, α_r (r-protein synthesis rate/total protein synthesis rate), was reduced; in the *rel*[−] strain the synthesis of stable RNA and r-protein was stimulated relative to the synthesis of total protein and α_r was increased. At more restrictive temperatures the coordination between the production of rRNA and r-protein in the *rel*[−] strain begins to deteriorate because of the severe inhibition of protein synthesis, although α_r continues to increase. Presumably relaxed particles are accumulated in these restrictive conditions⁸.

To elucidate further the nature of the coordinate expression of rRNA and r-protein genes in *rel*⁺ and *rel*[−] bacteria we have determined directly the amount of mRNA coding for r-protein during various degrees of amino acid deprivation. Partial or complete amino acid deprivation was accomplished by shifting

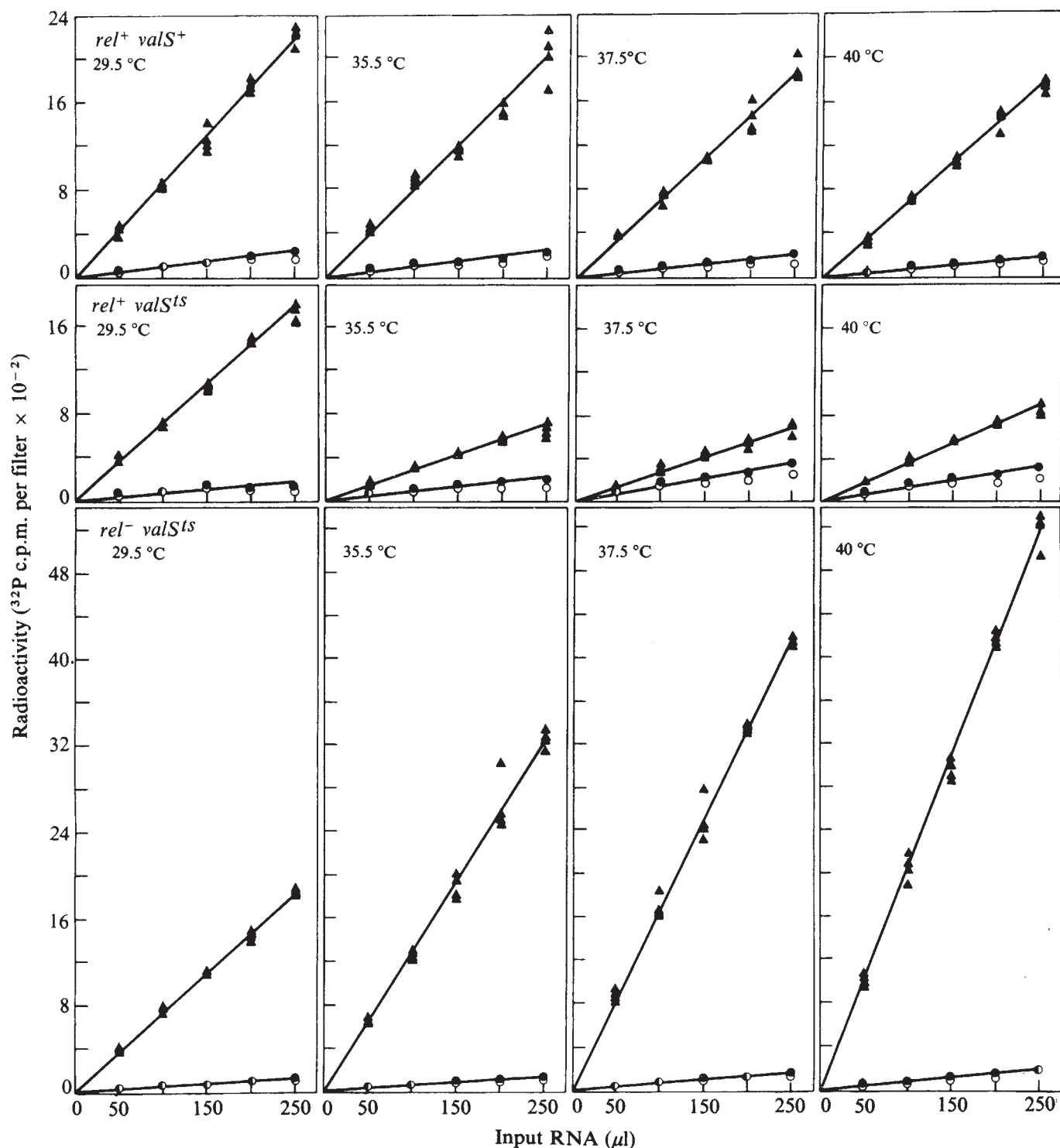


Fig. 1 Hybridisation of total cellular RNA to DNA from $\lambda dtrk$ and $\lambda dspc1$ transducing phages. The bacterial strains which require leucine were grown in minimal morpholinopropane sulphonic acid (MOPS)¹⁰ medium supplemented with 0.2% glucose, leucine ($20 \mu\text{g ml}^{-1}$), and 0.2 mM phosphate (specific activity 50 mCi mmol^{-1}) at 29.5°C in a rotary shaker bath for 20 cell doublings to label homogeneously the cellular nucleic acid. The doubling times of the bacterial mass in the cultures were monitored as absorbance at 460 nm and were between 65 and 70 min. At an A_{460} of 0.2–0.3, portions of the culture were shifted to rotary water baths set at 35.5 , 37.5 or 40°C . After 15 min the bacteria were collected and total cellular RNA was prepared. DNA was extracted from λ phages and transducing phages $\lambda dtrk$ and $\lambda dspc1$, denatured and immobilised on nitrocellulose membrane. The specific radioactivity of the RNA preparations was $1.44 \times 10^7 \text{ c.p.m. per } A_{260}$ and the concentration in $A_{260} \text{ U ml}^{-1}$ at the respective incubation temperatures of 29.5 , 35.5 , 37.5 and 40°C were: $valS^+rel^+$ (NF314) 1.06, 1.08, 1.08 and 1.12; $valS^{1s}rel^+$ (NF536) 1.11, 1.08, 1.10, 1.09; $valS^{1s}rel^-$ (NF537) 1.07, 1.08 and 1.05. RNA (50 – $250 \mu\text{l}$) was hybridised in a total volume of 2 ml of $2 \times \text{SSC}$ to two nitrocellulose filters containing no DNA, two filters containing $5 \mu\text{g}$ each of λDNA , two filters containing $5 \mu\text{g}$ each of $\lambda dtrk$ DNA and four filters containing $5 \mu\text{g}$ each of $\lambda dspc1$ DNA for 18 h at 67°C . After hybridisation, the filters were washed with $2 \times \text{SSC}$, treated with T_1 RNase and RNase A, and counted in 5 ml of toluene as scintillation fluid. Radioactivity associated with the blank filters ranged from about 50 to 250 counts per filter with increasing input RNA and were subtracted from the radioactivity associated with the λDNA filters ($\circ$), $\lambda dtrk$ DNA filters ($\bullet$) and $\lambda dspc1$ DNA ($\blacktriangle$) filters. The amount of specific hybridisation is obtained as four times the difference between the $\lambda dspc1$ and $\lambda dtrk$ curves. The factor four accounts for the four separate filters each containing $5 \mu\text{g}$ of $\lambda dspc1$ DNA in each hybridisation assay.

Table 1 Fraction of the total cellular RNA which is specific mRNA for r-protein coded for by genes on the λ dspc1 phage

Bacterial strain	Incubation temp. (°C)	Specific hybrids (c.p.m. $\times 10^{-3}$)	Input RNA (c.p.m. $\times 10^{-6}$)	Specific hybrids ($\times 10^3$)		Specific r-protein mRNA fraction (%)
				Input RNA		
<i>valS</i> ⁺ <i>rel</i> ⁺ (NF312)	29.5	7.85	3.82	2.06		0.275
	35.5	7.14	3.89	1.83		0.244
	37.5	6.85	3.89	1.76		0.234
	40.0	6.49	4.03	1.61		0.215
<i>valS</i> ^{ts} <i>rel</i> ⁺ (NF536)	29.5	6.66	4.00	1.67		0.223
	35.5	1.96	3.89	0.50		0.067
	37.5	0.91	3.96	0.23		0.030
	40.0	2.10	3.93	0.53		0.070
<i>valS</i> ^{ts} <i>rel</i> ⁻ (NF537)	29.5	6.29	3.85	1.63		0.217
	35.5	12.40	3.89	3.19		0.425
	37.5	15.95	3.89	4.11		0.548
	40.0	19.90	3.78	5.27		0.704

The radioactivity in specific hybrids was obtained from the experiments illustrated in Fig. 1 and is equal to four times the difference between radioactivity hybridised to λ dspc1 DNA and λ dtrk DNA. The factor four accounts for the four separate λ dspc1 DNA filters each containing 5 μ g of DNA. Values calculated for a total of 20 μ g λ dspc1 DNA are given in the table (see below). The calculation was made at an input of 250 μ l of RNA and the radioactivity in the input RNA was calculated from the information in the legend of Fig. 1. The fraction of the total RNA which is mRNA coded for by the r-protein genes carried in the λ dspc1 transducing phage was obtained using the relationship:

$$sK = H/R$$

where s is the fraction of specific r-protein mRNA; H is radioactivity in specific λ dspc1 hybrids; R is radioactivity in input total RNA; K is a constant ≈ 0.75 . The value of the constant K depends on the conditions of hybridisation and accounts for the fraction of the specific RNA which enters into hybrids with λ dspc1 at equilibrium. In our condition of hybridisation (20 μ g of λ dspc1 DNA per assay) we have found that the numerical value of K is between 0.75 and 0.90. The calculation is based on the lower estimate of 0.75 and may thus contain a systematic over-estimation of the fraction of specific r-protein mRNA by as much as 10–20% (our unpublished result).

isogenic *rel*⁺ and *rel*⁻ strains, carrying a temperature sensitive mutation in the *ValS* gene, from the permissive temperature of 29.5 °C to the semipermissive or restrictive temperatures of 35.5, 37.5 or 40 °C. The amount of mRNA coding for r-protein was determined by hybridisation of total cellular RNA, isolated from the bacterial strains after incubation at the various temperatures, with DNA from a lambda transducing phage carrying approximately half the r-protein genes from the bacterial chromosome⁹. These measurements clearly demonstrate that the stringent control system directly or indirectly regulates the amount of mRNA for r-protein as well as the rate of accumulation of stable RNA. This regulation may be exercised at the level of initiation of transcription at the promoters for the r-protein genes or alternatively the *rel* gene product may influence the stability of the mRNA for r-protein. Finally, we have also examined the nature of the residual protein synthesised at the restrictive temperature of 40 °C where inhibition of protein synthesis is virtually complete. The results indicate that r-protein represents a significant fraction of the residual protein synthesis in both the *rel*⁺ and *rel*⁻ strain and reflects the amount of r-protein mRNA detected by DNA-RNA hybridisation.

mRNA for r-proteins

Three isogenic strains were used: NF314, a non-temperature sensitive stringent strain (*leu*⁻*valS*⁺*rel*⁺, parent); NF536, a temperature sensitive stringent strain (*leu*⁻*valS*^{ts}*rel*⁺) and NF537, a temperature sensitive relaxed strain (*leu*⁻*valS*^{ts}*rel*⁻). Cultures were grown at the permissive temperature of 29.5 °C in glucose minimal medium containing ³²P-orthophosphate. Portions of the cultures were shifted to 35.5, 37.5 and 40 °C. In the temperature sensitive strains the protein synthesis rate was initially reduced by about 10%, 80% and greater than 95% at the three respective temperatures. The classical stringent and relaxed responses of stable RNA accumulation were elicited in the *rel*⁺ and *rel*⁻ cultures at these elevated temperatures (refs 3 and 7 and Fig. 3).

The effect of the stringent control system on the expression of r-protein genes was examined by hybridising the total cellular RNA with DNA prepared from bacteriophage λ or transducing phages λ dtrk or λ dspc1. The λ dtrk DNA carries the *aroE* and *trkA* genes from the bacterial chromosome but no

detectable r-protein genes, whereas the λ dspc1 phage carries the *aroE* and *trkA* genes in addition to approximately 22 r-protein genes from the cluster located near 64 min on the bacterial chromosome⁹. The r-protein genes on the λ dspc1 phage include S3, S4, S5 (*spc* gene product), S7, S8, S11, S13, S14, L5, L6, L11, L13, L14, L15, L16, L17, L18, L19, L22, L23, L24 and L30 and account for about 400,000 daltons of protein or about half of the aggregate protein per 70S ribosome.

Various amounts of the ³²P-RNA preparations were hybridised to a constant and excess amount of DNA from the bacteriophage λ , λ dtrk and λ dspc1 (Fig. 1). The amount of radioactivity entering into λ dtrk hybrids was only slightly greater than the nonspecific λ hybrids and suggests that the *aroE-trkA* region of the bacterial chromosome has a relatively low transcriptional activity. In contrast, at the permissive temperature the radioactivity entering into λ dspc1 hybrids was significantly greater than into the λ dtrk hybrids, indicating that the unique bacterial DNA carried by the λ dspc1 phage has a relatively high transcriptional activity. This transcriptionally active DNA represents r-protein genes and is virtually free of sequences which are homologous to stable RNA and other non-r-protein mRNAs (our unpublished results). Thus the difference between hybridisation to λ dspc1 and λ dtrk DNA has been termed specific r-protein mRNA and is homologous to the r-protein DNA region carried by the λ dspc1 phage.

The relative abundance of r-protein mRNA in the various RNA preparations is proportional to the slope of the specific hybridisation curves (Fig. 1 and Table 1). In the conditions of hybridisation used here, approximately 75–85% of the specific r-protein mRNA is in a DNA-RNA hybrid at equilibrium. Thus the absolute fraction of the total RNA preparation which is specific r-protein mRNA can be estimated (Table 1).

In the non-temperature sensitive parental strain the fraction of specific r-protein mRNA represents 0.21–0.24% of the total RNA and was essentially independent of the incubation temperature. In contrast, when the *valS*^{ts}*rel*⁺ strain was shifted to 35.5, 37.5 or 40 °C, the fraction of specific r-protein mRNA decreased from a value of about 0.22% at 29.5 °C to an apparent minimum value of about 0.03% at 37 °C; when the *valS*^{ts}*rel*⁻ strain was shifted to the respective temperatures, the fraction of specific r-protein mRNA gradually increased from a value of about 0.22% at 29.5 °C to a value of about 0.70% of the total RNA at 40 °C (Fig. 1 and Table 1). The

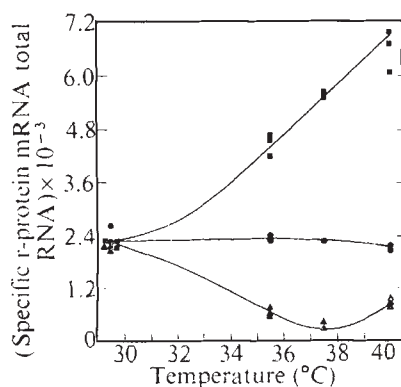


Fig. 2 Fraction of specific r-protein mRNA during partial or complete inhibition of valyl-tRNA aminoacylation. The *valS*⁺*rel*⁺ parental strain (●), the *valS*⁺*rel*⁺ strain (▲) and the *valS*⁺*rel*⁻ strain (■) were grown at the permissive temperature of 29.5 °C and shifted to semipermissive or restrictive temperature as described in Fig. 1. The fraction of the total RNA which is specific r-protein mRNA and homologous to the r-protein genes carried by the λ dspcl phage was determined after incubation at 29.5 °C or 15 min after a shift to 35.5, 37.5 or 40 °C as described in Fig. 1 and Table 1.

results from several identical experiments of this type are summarised in Fig. 2.

During steady-state growth at the permissive temperature, r-protein accounts for about 13% of the total protein synthesis rate in these conditions⁷ and the specific r-protein mRNA fraction is about 0.23% of the total cellular RNA. If all mRNA molecules are translated with equal efficiency and the r-protein genes carried by the λ dspcl phage code for 50% of the total protein per ribosome, the amount of total mRNA can be calculated to be about 3.5% of the total cellular RNA.

After shifts from the permissive temperature of 29.5 °C to the semipermissive temperature of 35.5 °C, the accumulation of stable RNA and individual r-proteins remains closely coupled⁷. In the *valS*⁺*rel*⁺ strain the rates of stable RNA and r-protein accumulation decrease to about 60% of control values at 29.5 °C whereas in the *valS*⁺*rel*⁻ strain the rates of accumulation increase to about 60% above the control values. Thus it was concluded that both the accumulation of stable RNA and r-protein are subject to the influence of the stringent control system⁷. The results summarised in Fig. 2 now show further that the stringent control system influences the expression of r-protein genes in these conditions by directly or indirectly regulating the amount of r-protein mRNA available for translation.

RNA, protein and r-protein synthesis

The presence of mRNA for r-proteins in the RNA prepared from the *rel*⁺ and *rel*⁻ strains incubated at the restrictive temperature of 40 °C suggests that r-protein should account for a significant fraction of the residual protein synthesised under these conditions. To verify this we have determined the accumulation rate of RNA and protein and the relative differential synthesis rates of individual r-proteins in these restrictive conditions. The exponential synthesis rates of stable RNA and protein after a shift to 40 °C are illustrated in Fig. 3. In the parental strain the rates of accumulation of stable RNA and protein both increased approximately 1.6–1.7-fold immediately after the shift. In contrast protein accumulation almost stopped in both the *valS*⁺*rel*⁺ and *valS*⁺*rel*⁻ strains at 40 °C. At the same time stable RNA accumulation was drastically reduced in the *valS*⁺*rel*⁺ strain and initially increased approximately 1.7-fold in the *valS*⁺*rel*⁻ strain. These strains thus illustrate the classical stringent and relaxed phenotypes after the inhibition of aminoacylation of valyl-tRNA^{3,7}.

The relative differential synthesis rates of individual r-proteins^{7,11} at the restrictive temperature were determined by prelabelling the strains with ¹⁴C-isoleucine at 29.5 °C. The

Table 2 Relative differential synthesis rates of individual r-proteins at the restrictive temperature of 40 °C

r-protein	<i>valS</i> ⁺ <i>rel</i> ⁺		<i>A</i> _i quotients		<i>valS</i> ⁺ <i>rel</i> ⁻	
	29.5 °C	40 °C	29.5 °C	40 °C	29.5 °C	40 °C
S3	0.96	0.90	0.97	0.78	0.97	1.92
S4	0.93	0.89	1.00	0.39	1.01	1.41
S5	0.93	0.89	0.98	1.13	1.01	1.67
S6	1.05	0.97	1.11	0.64	1.18	5.84
S7	0.96	0.86	0.99	0.29	1.05	1.43
S8	0.93	0.84	0.96	0.63	0.97	1.67
S9 + S11	0.96	0.91	0.95	0.27	1.02	2.85
S10	0.95	0.93	0.98	0.80	1.01	5.10
S12	0.98	0.73	1.05	0.37	1.10	2.83
S13	0.95	0.87	0.96	0.55	1.03	3.18
S14	0.97	0.80	1.02	1.27	1.04	2.86
S15	0.96	0.87	0.97	0.86	1.02	5.07
S16	0.99	0.93	0.97	0.19	0.99	1.58
S17	0.98	0.90	0.97	0.45	1.01	2.43
S18	0.92	0.80	0.93	0.30	0.98	1.17
S19	0.93	0.89	0.97	0.65	1.01	1.98
S20	0.94	0.93	0.96	0.39	1.00	3.87
S21	1.04	0.99	0.96	0.37	1.08	1.26
L1	0.96	0.89	0.99	0.26	1.02	4.00
L2	0.92	0.94	0.99	0.62	1.05	1.66
L3	0.96	0.85	0.98	0.26	0.98	2.49
L5	0.92	0.87	0.96	1.00	1.00	4.74
L6	0.92	0.90	0.98	0.93	0.96	1.21
L7	0.95	1.02	0.97	0.66	1.05	4.92
L10	0.94	0.90	0.94	0.65	1.00	5.23
L11	0.94	0.89	0.97	0.48	1.01	4.58
L12	0.87	0.76	0.91	0.15	0.97	3.55
L13	0.92	0.88	0.97	0.18	0.99	2.72
L14	0.98	0.91	0.96	1.09	0.93	1.25
L15	0.94	0.93	0.93	0.95	0.97	1.00
L16	0.96	0.97	0.96	1.52	0.96	2.25
L17	0.94	0.94	0.96	0.50	1.00	3.15
L18	0.96	0.89	0.93	1.32	1.02	1.76
L19	0.97	0.94	1.01	0.18	1.03	1.80
L20 + S12	1.00	0.98	0.99	0.27	1.09	1.28
L21	0.96	0.93	0.96	0.37	1.00	2.63
L22	0.94	0.92	0.96	1.14	1.06	1.52
L24	0.97	0.94	0.96	0.85	1.02	0.98
L27	0.98	0.92	0.99	1.01	0.98	2.38
L28	1.00	0.93	0.95	0.53	1.08	1.74
L30	0.96	0.97	0.97	2.49	1.01	2.38
L32	0.97	0.95	1.03	1.20	1.01	4.56
L33	0.97	0.96	0.97	2.36	1.06	2.17
L7 + L12	—	0.91	—	—	—	—
Mean	0.96	0.90	0.97	0.73	1.01	2.65
s.d.	0.03	0.06	0.03	0.52	0.04	1.36

Bacterial cultures were grown as described in Fig. 1 in medium containing non-radioactive phosphate. At an A_{490} of 0.1, a 10-ml portion of the culture was labelled with ¹⁴C-isoleucine (specific activity 306 mCi mmol⁻¹; 3 nmol ml⁻¹). When the A_{490} reached 0.3 the cultures were shifted to 40 °C and after 15 min, labelled with ³H-isoleucine (specific activity 30 Ci mmol⁻¹; 1 nmol ml⁻¹). After 5 min, labelling was terminated by addition of excess isoleucine, leucine and valine (3 μ mol ml⁻¹) and incubation continued for a further 5 min and then rapidly cooled to 0 °C. The control samples at 29.5 °C were labelled in a similar manner although the length of the ³H label was reduced to 1 min and the post-labelling incubation period increased to 30 min. Cells were collected and the incorporation of ³H and ¹⁴C into total protein was determined as described previously^{7,11}; the r-proteins were extracted directly from cell lysates and separated by the gel electrophoresis system of Kaltschmidt and Wittmann¹². The ³H and ¹⁴C radioactivity associated with each of the r-proteins was determined following oxidation and scintillation counting and the *A*_i values were calculated:

$$A_i = \frac{[^3\text{H}/^{14}\text{C}] \text{ in } i^{\text{th}} \text{ r-protein}}{[^3\text{H}/^{14}\text{C}] \text{ in total protein}} \\ = \frac{[^3\text{H}] \text{ in } i^{\text{th}} \text{ r-protein}/[^3\text{H}] \text{ in total protein}}{[^{14}\text{C}] \text{ in } i^{\text{th}} \text{ r-protein}/[^{14}\text{C}] \text{ in total protein}}$$

As the above relationships show, the *A*_i values give the relative change in the differential synthesis rates of the individual r-proteins after the shift to 40 °C. The control values at 29.5 °C are equal to approximately 1.0. These *A*_i values are independent of the recoveries of the individual r-protein during extraction and electrophoresis because of normalisation with the ¹⁴C prelabel. Furthermore, detection does not require incorporation of the proteins into ribosomal particles since the r-proteins are extracted directly from the whole cell lysates. Detection, however, requires maturation of r-proteins so that their electrophoretic properties are identical to the ¹⁴C-labelled r-proteins.

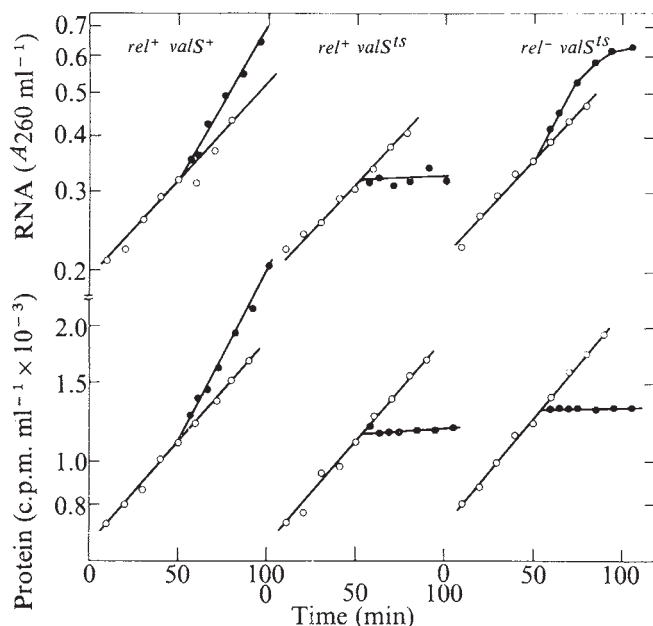


Fig. 3 Accumulation of RNA and protein after a temperature shift from 29.5 to 40 °C. The accumulation of RNA (upper curves) and protein (lower curves) was monitored in the *valS¹⁵rel⁺* parent (NF314), the *valS¹⁵rel⁺* strain (NF536) and the *valS¹⁵rel⁻* strain (NF537) at 29.5 °C (○) and after a shift of a portion of the culture to 40 °C (●). Growth conditions are described in Fig. 1; the growth medium contained 0.2 mM non-radioactive phosphate and radioactive leucine (20 µg ml⁻¹). The temperature shifts occurred at 50 min on the time scales. RNA was measured as A_{260} of acid insoluble, alkali labile material and protein was measured in the three leucine requiring strains as long term ¹⁴C-leucine (281 mCi mmol⁻¹, 0.1 µCi ml⁻¹) incorporation into acid insoluble material⁷.

cultures were shifted to 40 °C and, after 15 min, were exposed to ³H-isoleucine for 5 min. Incorporation was terminated by addition of excess non-radioactive isoleucine and incubation was continued for a further 5 min to allow time for completion and maturation of nascent r-protein chains and to minimise the possibility of proteolytic degradation of mature ³H-labelled r-protein in the temperature sensitive strains. The [³H/¹⁴C] isotope ratios in total cellular protein and 43 separate r-proteins (extracted from whole cell lysates and separated electrophoretically^{7,11,12}) were determined and the quotients of the respective isotope ratios were calculated (Table 2). These A_i quotients illustrate the relative change in the differential synthesis rates of the individual r-proteins following the shift from 29.5 to 40 °C.

At the permissive temperature of 29.5 °C, total r-protein synthesis accounts for approximately 13% of the total protein synthesis rate (that is, $\alpha_r = 0.13$)⁷. When the parental strain was shifted to 40 °C, the A_i quotients remained essentially constant and equal to the unshifted control values of approximately 1.0 (Table 2). This indicates that the temperature shift has little or no effect on the balanced synthesis of stable RNA, total protein and individual r-proteins in the non-temperature sensitive parental strain and r-protein continues to account for about 13% of the total protein synthesis rate. This observation correlates with the apparent constancy in the fraction of specific r-protein mRNA in the parental strain (Fig. 2).

In contrast, when the *valS¹⁵* strains were shifted to 40 °C a non-coordinate response of the A_i quotients was observed and indicates an imbalance in the production of individual r-protein species (Table 2). In the *valS¹⁵rel⁺* strain the differential synthesis rates of some r-proteins increased as much as 2.5-fold (that is, $A_i > 1.0$) while most decreased up to fivefold (that is, $A_i < 1.0$). The average of the 43 A_i quotients was 0.73. Thus, at the restrictive temperature of 40 °C, complete and mature r-proteins account for about 9% of the residual amino acid

incorporation although individual r-proteins are synthesised in drastically disproportionate amounts.

In the *valS¹⁵rel⁻* strain the differential synthesis rates of individual r-proteins increased up to fivefold or more (A_i values between 1.0 and 5.84; Table 2). The average of the 43 individual A_i quotients was 2.65. This indicates that r-protein accounts for about 35% of the residual protein synthesis at 40 °C and again individual r-proteins are produced in drastically disproportionate amounts.

During inhibition of protein synthesis by amino acid deprivation, the chain elongation rate of nascent peptides is reduced and the probability for release of unfinished chains from the ribosome is increased^{13,14}. The premature release probability increases with molecular weight and with the content of deprived amino acid, and released fragments are rapidly degraded. Thus, the imbalance observed in the synthesis rates of individual r-proteins at the restrictive temperature may in part result from differences in their molecular weights and valine content. Other processes such as degradation of mature r-proteins which are not incorporated into ribosomal particles¹⁵ or accumulation of precursor forms of r-proteins¹¹ which escape detection in the electrophoresis system used¹² would also contribute to the non-coordinate production and result in underestimates in synthesis rates of individual r-proteins. In the experimental procedure used here, a 5-min chase following the ³H-isoleucine was provided to enable completion of labelled nascent r-proteins and subsequent maturation and to minimise the degradation of mature r-protein. The possibility remains, however, that at least some of the non-coordinate production of r-proteins may be a direct effect of the mechanism controlling the expression of r-protein genes.

Goodman *et al.*^{16,17} previously observed that only one or a few 50S r-proteins accounted for the major portion of the residual protein synthesis in an amino acid deprived *rel⁻* strain whereas all r-proteins were produced in nearly proportionate amounts and represented a very small fraction of the residual protein synthesis in a *rel⁺* strain. The difference between our results and the earlier work of Goodman *et al.*^{16,17} may be explained by the fact that their detection of r-proteins labelled during amino acid deprivation required readdition of the deprived amino acid and subsequent incorporation of the radioactive r-proteins into ribosomal particles. They made no attempt to determine the stability of the free labelled r-proteins and the radioactive proteins associated with the isolated ribosomes were analysed by one-dimensional gel electrophoresis. In our experiments radioactive r-proteins were extracted directly from whole cell lysates within minutes of the termination of the labelling period; incorporation of labelled r-protein into ribosomal particles was not required and labelled r-proteins were separated using the standard two-dimensional electrophoresis system of Kaltschmidt and Wittmann¹².

Regulation of r-protein gene expression

Clear and distinct differences are apparent in the amount of specific r-protein mRNA in *rel⁺* and *rel⁻* strains during partial or complete inhibition of valyl-tRNA aminoacylation. The physiological significance of these differences is substantiated by the observation that the synthesis rates of rRNA and r-protein remain in balance at semipermissive temperatures up to at least 35.5 °C⁷. That is, during partial inhibition of protein synthesis the expression of rRNA and r-protein genes is coordinately reduced in the *rel⁺* strain and coordinately enhanced in the *rel⁻* strain.

The synthesis of mRNA in general has been thought to be independent of the stringent control system^{6,8}; the amounts of tryptophan^{6,18} and β -galactosidase mRNA¹⁸ were observed to be nearly equal in *rel⁺* and *rel⁻* strains during amino acid deprivation and the half lives of these mRNAs as well as total mRNA were estimated to be essentially identical to the steady state values of 1–2 min^{5,6,18,19}. Our results show that the

regulation of synthesis of r-protein mRNA is subject to the influence of the stringent control system and is different from that of mRNAs studied previously. In this connection, the experiments of Lazzarini and Winslow⁵ should be noted. They observed that the synthesis of total mRNA was reduced to 40% and 65% in the *rel*⁺ and *rel*⁻ strains respectively during the initial 20–40 min after amino acid deprivation. At the same time stable RNA accumulation was reduced to about 10% in the *rel*⁺ and essentially unaffected in the *rel*⁻ strain whereas residual protein synthesis in both strains was reduced to about 5% of the control rate. Thus, the excess synthesis of total mRNA observed in the *rel*⁻ strain relative to the *rel*⁺ strain during amino acid deprivation may reflect to a large extent the increased synthesis of r-protein mRNA we observed (Fig. 2). Furthermore, these observations seem to indicate that the regulation of r-protein mRNA by the stringent control system is exerted at the level of initiation of transcription rather than at the level of mRNA stability. We are investigating this possibility.

We observed that the r-protein mRNA fraction in the *val*^S*rel*⁺ strain begins to increase at temperatures above 37 °C (Fig. 2, lower curve). This may be of physiological significance and related to the general mechanism controlling the transcription of r-protein genes. Lazzarini and Winslow⁵ observed that the residual stable RNA accumulation (10%) is greater than the residual total protein accumulation (5%) in the *rel*⁺ strain during amino acid starvation. It is possible that the transcription of r-protein genes is negatively regulated by free r-proteins (that is autogenous regulation^{20,21}) such that any rRNA produced in excess during severe amino acid deprivation would deplete the pools of free r-protein and

consequently stimulate the transcription of r-protein genes in the *rel*⁺ strain. This is in fact observed at temperatures above 37 °C. During partial or complete inhibition of protein synthesis in the *rel*⁻ strain, RNA always accumulates in excess of total protein and the expression of r-protein genes is enhanced (Fig. 2 and ref. 7). Other explanations are also possible, however, and further experiments are required to test the validity of the specific mechanism suggested here.

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- ¹ Maaløe, O., *Devl Biol. Symp.*, **3**, 33–58 (1969).
- ² Kjeldgaard, N. O., and Gausing, K., in *Ribosomes* (edit. by Nomura, M., Tissières, A., and Lengyel, P.), 369–392 (Cold Spring Harbor Laboratory, New York, 1974).
- ³ Stent, G., and Brenner, S., *Proc. natn. Acad. Sci. U.S.A.*, **47**, 2005–2014 (1961).
- ⁴ Edlin, G., and Broda, P., *Bact. Rev.*, **32**, 206–226 (1968).
- ⁵ Lazzarini, R., and Winslow, R., *Cold Spring Harb. Symp. quant. Biol.*, **35**, 383–390 (1970).
- ⁶ Lavalle, R., and De Hauwer, G., *J. molec. Biol.*, **37**, 267–288 (1968).
- ⁷ Dennis, P. P., and Nomura, M., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3819–3823 (1974).
- ⁸ Nakada, D., Anderson, I., and Magasanik, B., *J. molec. Biol.*, **9**, 472–488 (1964).
- ⁹ Jaskunas, S. R., Lindahl, L., and Nomura, M., *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- ¹⁰ Neidhardt, F., Bloch, P., and Smith, D., *J. Bact.*, **119**, 236–247 (1974).
- ¹¹ Dennis, P. P., *J. molec. Biol.*, **88**, 25–41 (1974).
- ¹² Kaltschmidt, E., and Wittmann, H., *Analyt. Biochem.*, **36**, 401–412.
- ¹³ Brunschide, H., and Brener, H., *J. molec. Biol.*, **57**, 35–57 (1971).
- ¹⁴ Engbaek, F., Kjeldgaard, N. O., and Maaløe, O., *J. molec. Biol.*, **75**, 109–118 (1973).
- ¹⁵ Dennis, P. P., *Molec. gen. Genet.*, **134**, 39–47 (1974).
- ¹⁶ Goodman, D., Manor, G., and Rombants, W., *J. molec. Biol.*, **40**, 247–260 (1969).
- ¹⁷ Goodman, D., *J. molec. Biol.*, **51**, 491–499 (1970).
- ¹⁸ Edlin, G., Stent, G., Baker, R., and Yanofsky, C., *J. molec. Biol.*, **37**, 257–268 (1968).
- ¹⁹ Morris, D., and Kjeldgaard, N. O., *J. molec. Biol.*, **31**, 145–148 (1968).
- ²⁰ Goldberger, R., *Science*, **183**, 810–816 (1974).
- ²¹ Savageau, M., *Nature*, **252**, 246–249 (1974).

letters to nature

Lunar occultation of the hard X-ray source in the Crab Nebula

HARD X rays from the Crab Nebula were observed at a lunar occultation on 24 January 1975 with two sets of scintillation counters on board two balloons launched from Hyderabad (17°20'N, 78°27'E). The result obtained by one of the balloon observations is reported here.

The balloon payload comprised eight counters, each consisting of an NaI (TI) crystal (125 mm diameter × 3 mm thick) with the field of view of 30° cone defined by a cylindrical Pb–Sn collimator with vertical axis. Pulse heights of individual counters were telemetered event by event covering an energy range 20–70 keV. The balloon was floating at the ceiling altitude of 590 Pa (5.9 mbar) when an immersion of the X-ray source took place at about 16 h 43 min UT. The Crab Nebula was located at zenith angle 11°. The Crab pulsar was occultated by the Moon at position angle 102° and at a speed of 0.36" s⁻¹.

The counting rate averaged for every 10 s is shown in Fig. 1. The transition of the counting rate is clearly observed at about 16 h 43 min UT. The counting rate before the immersion can be represented by a linear relationship $C_1(t) = 284.5 - 0.00533(t - t_1)$ counts s⁻¹, where $t_1 = 16$ h 41 min UT and $t < t_1$ is the time (s); the relationship after the immersion is $C_2(t) = 255.9 - 0.00171(t - t_2)$ counts s⁻¹, where $t_2 = 16$ h 45 min UT. A

decrease before the immersion is associated with a decrease of the elevation angle of the Crab Nebula with time which results in a decrease of the effective area of the counters; the time dependence thereafter is associated with a slow descent of the balloon.

Referring to the standard 10 kHz time signal, we found the apparent period of the Crab pulsar to be 33.17582 ms which is consistent with the barycentric period of 33.1736 ms obtained from the radio observation at Arecibo, as informed by R. Payne. The pulse profile shows two distinct peaks as shown in Fig. 1, and the counts during 3 ms under each peak unambiguously represent the time dependence of the pulsed X-ray counting rate. The counts in every 10 s thus defined for the pulsar component are also shown in Fig. 1. There is a sharp drop at $t_p = 16$ h 43 min UT, which is slightly shifted from the centre of the transition region in the total counting rate. The counting rate resulting from the total pulsar component (f) is obtained from the pulse profile as $C_p = 7.8$ counts s⁻¹ which is 26.5% of the total counting rate of the Crab X rays.

Fixing $C_1(t_1)$, $C_2(t_2)$, C_p and t_p , we fitted a Gaussian model of the diffuse source to the observed counting rates between t_1 and t_2 . A minimum of $\chi_m^2 = 20.8$ (d.f. = 20) is obtained for the centre of the diffuse source $t_c = 16$ h 42 min 44 s⁺¹¹_{-9.5} s UT and the FWHM D_t in units of time is 95 s⁺⁴⁸₋₃₈ s, where the errors indicate the value of one parameter to be $\chi_m^2 + 1$

whereas the other is optimised. Note that for $t_c = t_p$ the minimum value of χ^2 is 22.8 for $D_t = 84$ s.

The time profile of the intensity for this model is shown in Fig. 1. The centre of the diffuse source is offset from the pulsar by $d = 6'' \pm 4''$ and the Gaussian width is $D = 34'' \pm 17''$. The former is as large as the angular distance between the pulsar and Wisp 1, whereas the latter contains the pulsar, four Wisps and Anvil, as shown in Fig. 2.

The observed data were also fitted to an alternative model that assumes two essentially line sources and the pulsar in between. A minimum of $\chi_m^2 = 17.2$ is obtained for the relative intensities of these sources and their positions: 48% (16 h 42 min 30 s), 26.5% (16 h 43 min 00 s = pulsar), 25.5% (16 h 43 min

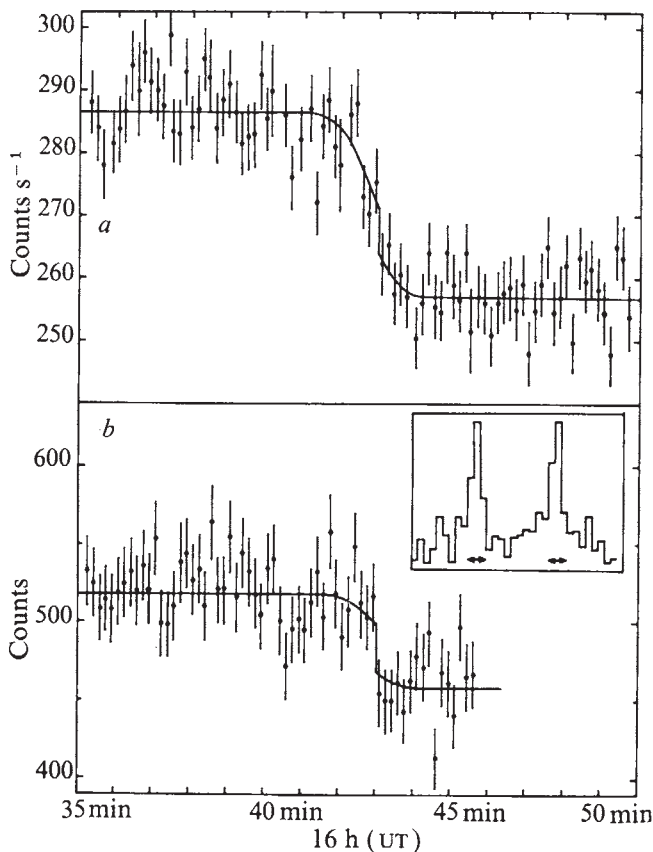


Fig. 1 The total counting rate (a) and the counts in 10 s bins for the pulsation peaks as indicated by arrows in the insert (b). The solid curve in the upper figure represents the total counting rate expected for the Gaussian diffuse source of FWHM of 95 s and centroid displaced by 16 s from a point source. The contribution of the point source (pulsar) relative to the total Crab X rays is 26.5%, judged from the pulse profile assuming no steady emission from the pulsar, and the contribution of the pulsation peaks as indicated in the insert is 51% of the total emission. The solid curve in the lower figure represents the counts in 10 s bins with phase corresponding to the peaks.

20 s). The first line source is located on the Wisps, whereas another is on the Anvil on the other side of the pulsar.

The average energy of hard X rays (~ 50 keV) and the position angle of 102° found are close to the situation for the immersion in the MIT experiment¹. Our result is compared with their Fit 2 $f = 25 \pm 16\%$, $d = 11'' \pm 4''$ and $D = 23'' \pm 6''$ at p.a. 130° . The size of the diffuse source is significantly smaller than that found in the keV region²⁻⁴. Further detail will be described elsewhere.

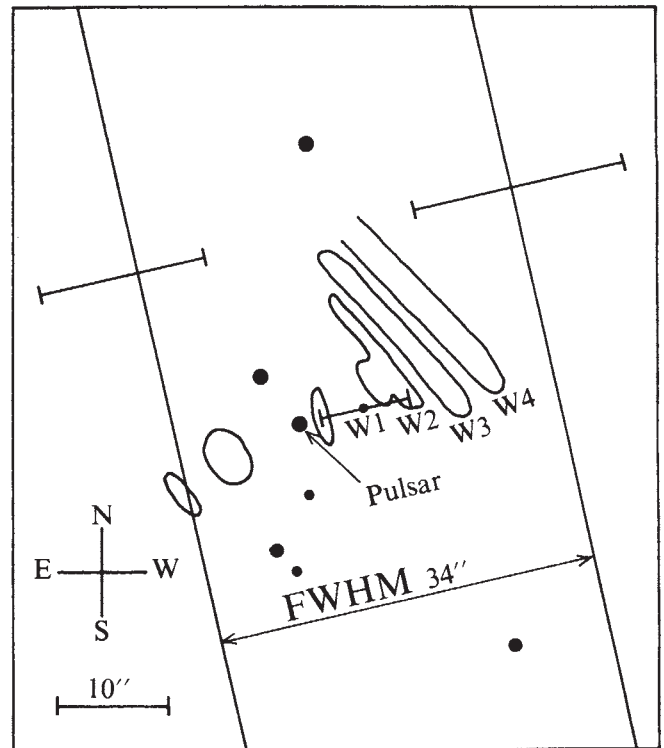


Fig. 2 X ray emitting region superimposed on the optical features of the Crab nebula given by Scargle and Pacini⁵. Wisps are indicated by W1, ..., W4; the Anvil is represented by two bright features on the east side of the pulsar. The centroid of the Gaussian diffuse source and FWHM, with their error bars, are also shown. Note that the error bars for the boundaries of the diffuse source are obtained from $\chi_m^2 + 1$ for parameters $t_c \pm D_t/2$.

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- ¹ Ricker, G. R., et al., *Astrophys. J. Lett.* (in the press).
- ² Bowyer, S., Byram, E. T., Chubb, T. A., and Friedman, H., *Science*, **146**, 912 (1964).
- ³ Oda, M., et al., *Astrophys. J. Lett.*, **148**, L5 (1967).
- ⁴ Davison, P. J. N., Culhane, J. L., and Morrison, V. L., *Nature* **253**, 610 (1975).
- ⁵ Scargle, J. D., and Pacini, F., *Nature phys. Sci.*, **232**, 114 (1971).

Variability and circular polarisation in the nucleus of NGC5128 at 10.7 GHz

THE galaxy NGC5128 (Centaurus A), has been found to contain a compact radio source in its nucleus¹. This compact source is one of the most remarkable yet discovered, and, as NGC5128 is the nearest of the strong radio galaxies (~ 4 Mpc), it provides a possibly unique opportunity to investigate the physical processes which produce these objects. The results which we report here place important constraints on its structure and magnetic field strength.

We have reported² that the 10.7-GHz flux density of NGC 5128 is substantially greater than those previously measured¹ at 2.7 and 8.1 GHz. If the radio spectrum is interpreted as arising from a single optically thick source of synchrotron radiation, then the high turnover frequency plus a lower limit³ of 0.001 arc s on the angular size at 2.3 GHz implies the presence of a strong magnetic field (≥ 10 gauss). Recently 31.5 and 89 GHz flux densities several times greater than the flux we measured at 10.7 GHz have been reported⁴; at these frequencies the source seems to vary by $\sim 50\%$ on a time scale of a day or less. The source has also been detected at 1.4 GHz (J. O'Sullivan, unpublished) and an upper limit has been placed on its flux at 0.4 GHz (B. Mills, unpublished); the radio flux densities including our results are summarised in Table 1. Centaurus A is a source of X rays^{5,6} which are believed to originate in the nuclear region⁷. Outside the nucleus it is probably⁸ an emitter of very high energy gamma rays ($h\nu > 10^{11}$ eV).

The traditional interpretation of compact radio sources is that clouds of relativistic electrons radiate by the synchrotron

Table 1 Radio fluxes for the nucleus of NGC5128

Frequency (GHz)	Flux density (Jy)
0.4	< 20
1.4	5.0
2.7	2.4
8.1	2.9
10.7	4–8 (variable)
31.5	20–25
89	15–25 (variable)

mechanism. Any homogeneous synchrotron source becomes optically thick below some frequency ν_{max} where the flux density is S_{max} . The magnetic field, B , and angular diameter, θ , are related by⁹

$$B = 1.3 \times 10^{-36} \theta^4 \nu_{\text{max}}^5 S_{\text{max}}^{-2} \quad (1)$$

where B is in gauss, θ in arc s, ν_{max} in Hz, and S_{max} in Jy. It is expected that there will also be high frequency emission from the relativistic particles by either the synchrotron or inverse Compton mechanisms or both, and this is proportional to $\theta^{-2} B^{-(1+\alpha)}$ where α is the high frequency spectral index¹⁰. So it seems possible to determine both angular diameter and magnetic field strength from combined observations of a source's spectrum at radio, optical, X-ray and gamma-ray wavelengths¹⁰.

But it is also possible to measure angular diameters directly by very long baseline interferometry, and the degree of circular polarisation provides an independent measure of the magnetic field strength; it is proportional to $(\nu^{-1} B \sin \phi)^{1/2} \cot \phi$ where ν is the observing frequency and ϕ the angle between the magnetic field and the line of sight. Furthermore, if radiation at two different frequencies arises from the same ensemble of relativistic particles, the time scales for any variability observed at those frequencies should be the same in the optically thin regime; if the component is optically thick, however, they are related in a

Table 2 10.7-GHz time variations in the nucleus of NGC5128

Date	10.7-GHz flux density (Jy)
June–July 1973	4.4
May 1974	8.1
January 1975	7.4

way which depends on the detailed operation of the mechanism which produces the variability.

We have observed the nucleus of NGC5128 in May 1974, and on two consecutive nights in January 1975. All observations were made with the Stanford University five element radio telescope¹¹. An attempt to detect circular polarisation was made during the January 1975 observations; in May 1974 only the 10.7-GHz flux density was measured.

The nucleus of NGC5128 is probably variable at 10.7 GHz; however, the time scale for this variability may be significantly longer than that reported at millimetre wavelengths⁴. Although we must observe Centaurus A (declination -43°) at elevations just above the horizon, its nature is such that we can use the Stanford interferometer to make relative measurements of the flux density of the compact central component from one epoch to another free from any systematic errors that might arise because of calibration difficulties. This is because the inner radio lobes of Centaurus A fall partially within our field of view, and we are able to compare the measured intensity of the central core to that of selected points in those lobes both to its east and west. The central source increased in strength by the same amount relative to the selected points on its opposite sides, some 85% ($\sim 8\sigma$), between July 1973 and May 1974, but changed little between then and January 1975 (Table 2). The relative intensities of the points in the lobes, however, remained constant to within 15% (1.5 σ) between the various dates in question. It would be difficult to explain such a large intensity change affecting only the compact central source by an instrumental effect. On the other hand, we observed the source on six days in 1973 and on two consecutive nights in 1975 and found no evidence for intensity changes of the order of 50% from one day to the next, as reported at millimetre wavelengths. So it seems that the time scale of variability in NGC5128 is significantly different at 10.7 GHz from that at frequencies between 30 and 90 GHz.

We failed to detect any circularly polarised flux and from the fluctuations of the measured crossed-horn visibilities we calculate a 5σ upper limit of 2.5% to the fraction which might be present.

Our circular polarisation limit probably allows us to rule out one-component models for the nucleus and place important constraints on two-components models. When source variability is considered, the 1.4-GHz flux density might be reconciled with the spectrum of a single source, opaque below 30 GHz; however, the magnetic field in such a source would be ~ 50 gauss and the resulting degree of circular polarisation at 10.7 GHz would be 25% for a field making an angle of 45° to the line of sight. Grindlay¹⁰ has proposed a model in which all the electromagnetic radiation emitted by the nucleus arises from two Compton-synchrotron emitting components, one opaque below 1 GHz with $\theta = 9 \times 10^{-3}$ arc s and $B = 0.01$ gauss, the other opaque below 30 GHz with $\theta = 4 \times 10^{-4}$ arc s and $B = 2$ gauss. In this model the smaller component dominates the emission at 10.7 GHz and requires 5% circular polarisation there if the angle between the magnetic field and line of sight is again 45° . Alternatively, such a component with a 2-gauss field can be reconciled with our limit on the degree of 10.7-GHz circular polarisation only if that angle is 10° or less. But the precise values of magnetic field and angular diameter in Grindlay's model are sensitive to the turnover frequencies assumed and the slope of the spectrum between 10^{19} and 10^{26} Hz. These are poorly determined by the data which, within their uncertainty, could

allow a two-component structure with magnetic field somewhat less than 1 gauss for the more compact component, and this could be reconciled with our circular polarisation limit.

Our limit on circular polarisation is not sufficiently stringent even to rule out the highly idealised model of Grindlay¹⁰, given the uncertainties in the other observed parameters. Condon and Dressel¹² have discussed models in which there is a radial change in the particle density and field strength and radio variability at low frequencies is caused by expanding shock fronts. Since the accuracy of our circular polarisation measurement can be improved by an order of magnitude, and repeated at other frequencies, it would be valuable to fit such models to the spectrum of NGC5128 and calculate in detail the expected degree of circular (and linear) polarisation as a function of frequency.

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- ¹ Wade, C. M., Hjellming, R. M., Kellermann, K. I., and Wardle, J. F. C., *Astrophys. J. Lett.*, **170**, L11 (1971).
- ² Price, K. M., and Stull, M. A., *Nature phys. Sci.*, **245**, 83 (1973).
- ³ Broderick, J. J., Kellermann, K. I., Shaffer, D. B., and Jauncey, D. L., *Astrophys. J.*, **172**, 299 (1972).
- ⁴ Kellermann, K. I., *Astrophys. J. Lett.*, **194**, L135 (1974).
- ⁵ Bowyer, C. S., Lampton, M., Mack, J., and de Mendonca, F., *Astrophys. J. Lett.*, **161**, L1 (1970).
- ⁶ Kellogg, G., et al., *Astrophys. J. Lett.*, **165**, L49 (1971).
- ⁷ Tucker, W., Kellogg, E., Gursky, H., Giacconi, R., and Tananbaum, H., *Astrophys. J.*, **180**, 715 (1973).
- ⁸ Grindlay, J. E., Helmken, H. F., Hanbury Brown, R., Davis, J., and Allen, L. R., *Astrophys. J. Lett.*, **197**, L1 (1975).
- ⁹ Sligh, V. I., *Nature*, **199**, 682 (1963).
- ¹⁰ Grindlay, J. E., *Astrophys. J.* (in the press).
- ¹¹ Bracewell, R. N., et al., *Proc. IEEE*, **61**, 1249 (1973).
- ¹² Condon, J. J., and Dressel, L. L., *Astrophys. Lett.*, **15**, 203 (1973).

Excess spreading axes and spreading rate in Iceland

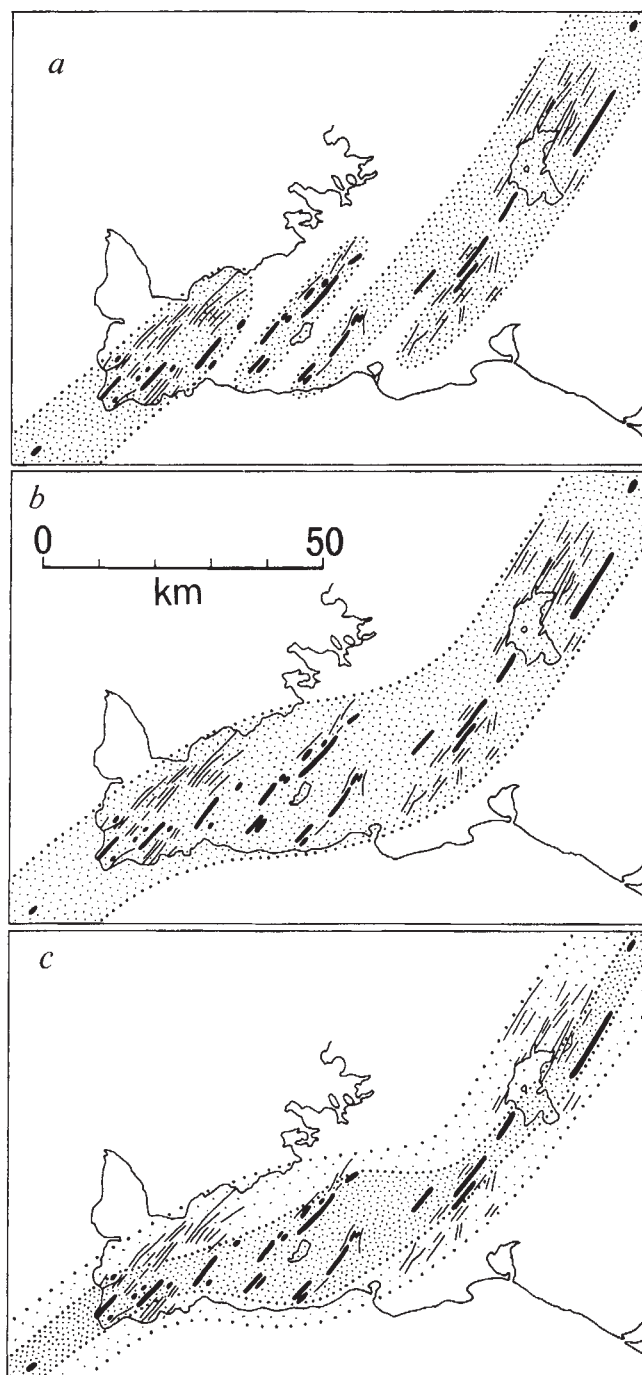
RIFTING and volcanic activity in Iceland is believed to be related to two active rift zones. I suggest, however, that several additional zones, some having a limited life, also exist. This concurs with a view¹ that the active zones are not immutable. It is now becoming increasingly apparent, moreover, that the southern half of Iceland has a spreading rate several times greater than that of both the northern half and some submerged parts of the Mid-Atlantic Ridge.

The term 'axial rift zone' is applied here to a zone of crustal spreading in which fissures (both eruptive and non-eruptive) are surface expressions of the rifting and are accompanied by axial subsidence. Axial rift zones are further classed as 'active' if they contain postglacial fissures or eruptive centres (active within about the past 10 kyr), 'dormant' if they lack postglacial fissures but contain young moberg (intraglacial hyaloclastite and pillow lava) mountains or young interglacial volcanoes formed towards the end of the Ice Age, and 'extinct' if they lack young, constructional volcanic features. An extinct rift zone is marked by a dyke swarm along the axis of a syncline in which the dykes are the sub-surface expression of both the eruptive and non-eruptive fissures.

The active rift zones can be delineated in different ways (Fig. 1a and b) according to the scale on which the fissure

distribution is examined. In Fig. 1a the edges of active zones have been drawn near the outermost postglacial fissures and the continuity of zones is extrapolated across areas where fissures may be concealed by ice, water, or recent sedimentary and volcanic accumulations. Figure 1b shows a broader interpretation and may perhaps approach more closely the fundamental underlying structure (Fig. 1c). There is a narrow median zone within which magma is generated and erupts at the surface, and narrow marginal zones which have fissures but in which magma fails to reach the surface. It may be that

Fig. 1 Fissure distribution in south-western Iceland; a and b, two differing views of the configuration of the rift zone (stippling, active rift zone; thick lines, eruptive fissures and vents; thin lines, non-eruptive fissures); c, a median zone (close stippled) in which magma reaches the surface, and marginal zones (open stippled) in which it does not. Interpretation a is believed to express most satisfactorily the structural relationships, and c the thermal relationships, in the rift zone.



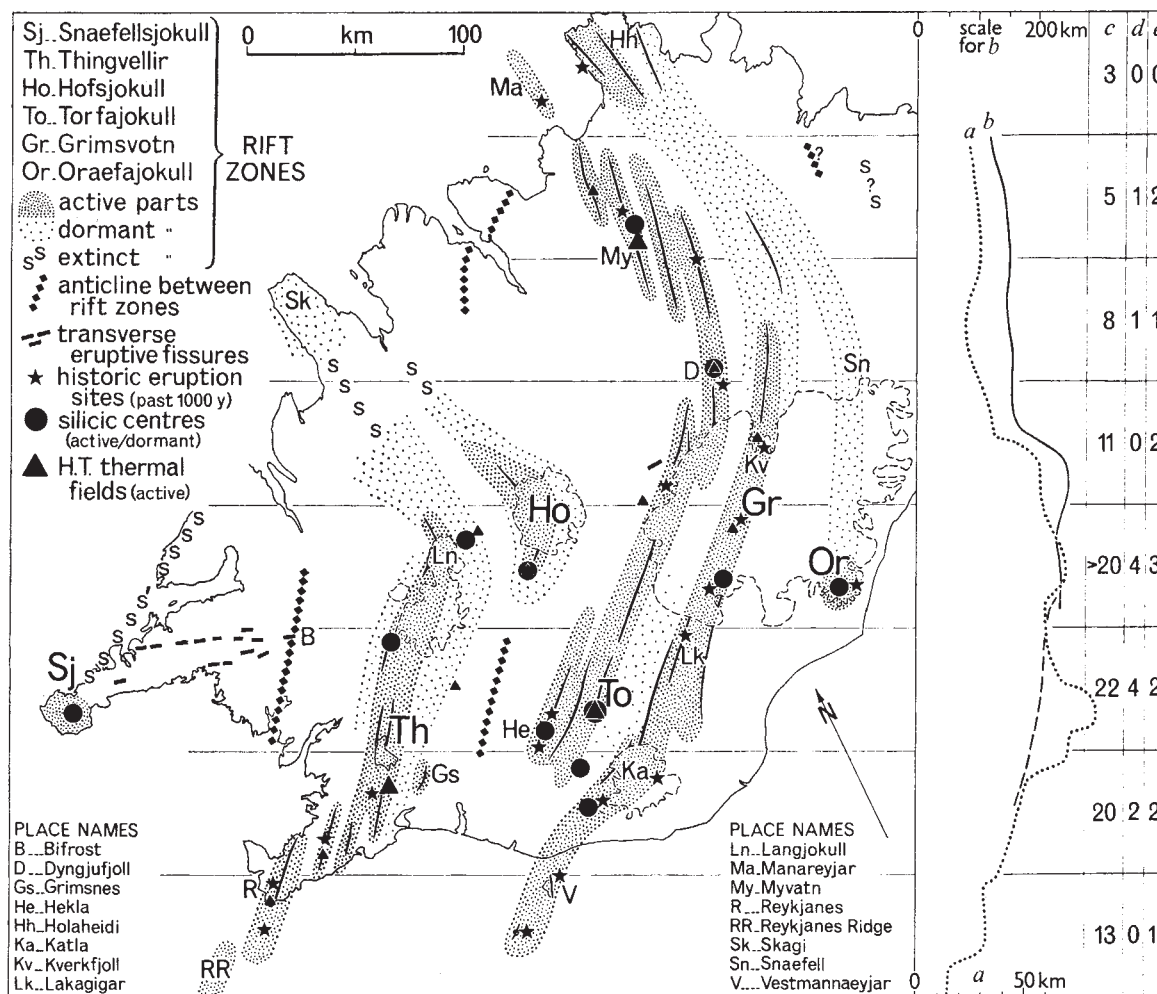


Fig. 2 The distribution of rift zones in Iceland. Curve *a*, aggregate width of active rift zones; *b*, aggregate width of outcrop of rocks younger than 3 Myr old; *c*, number of recorded eruptions in the past 1,000 yr; *d*, number of active and dormant silicic volcanic centres; *e*, number of active high temperature thermal fields. (In every case the number is taken for each of the eight strips drawn across Iceland in the presumed spreading direction.)

magma is generated only in the median zone and when it moves sideways into the marginal zones it fails to reach the surface there because of its lower volumetric rate of rising, caused by sideways flow.

The separate areas of fissures in south-western Iceland (Fig. 1a) are regarded as offset sectors of a single rift zone arranged *en echelon* and related to a continuous median zone of magma generation at depth. The offset sectors themselves trend parallel to the fissure direction. I do not accept the view that the sectors have been offset by north-westerly trending faults². A similar but less clear *en echelon* arrangement is seen in the Myvatn-Manareyjar area (Fig. 2). It is supposed that the amount of spreading in a given time is constant along any line drawn across the *en echelon* sectors in the spreading direction.

I suggest that the active zone around Hekla and Myvatn (Fig. 2), previously regarded as a single broad zone, in fact comprises a pair of narrow, active rift zones separated by a 20-km strip within which there is no postglacial activity. The eastern zone of the pair is the Grimsvotn Zone (Fig. 2) which is dormant in its northern part although it is believed to extend to Hólaheidi where, for a stretch, it is again active. The western zone of this pair is the Torfajökull Zone (Fig. 2) which is active along its entire length, with *en echelon* offsets both south and north of Dyngjufjöll. In places, this zone may itself be doubled, notably at Hekla, but also at Myvatn.

Another proposed rift zone is the Oraefajökull Zone (Fig. 2) which is dormant along most of its length and is active only

at its southern end. Young moberg mountains occur at, and north of, Snaefell, and intraglacial eruptions beneath the ice sheet between Oraefajökull and Snaefell have given rise to the hyaloclastic lava flows which formed under some valley glaciers in south-eastern Iceland³. The dormant northern continuations of the Grimsvotn and Oraefajökull zones have been broadly delineated (Fig. 1b) pending more detailed studies of the young moberg mountains in this area.

The Thingvellir Zone (Fig. 2) has a sharp inflection at Langjökull⁴, and the northward continuation to Skagi is dormant or extinct. The Hofsjökull Zone seems to be separate, with a dormant or extinct northward continuation towards Skagi. The final rift zone is that of Snaefellsjökull (Fig. 2), active only at its south-western end and extinct elsewhere.

The aggregate width of these active zones, measured across Iceland along parallel lines trending in the presumed spreading direction (Fig. 2a), reaches a maximum of about 80 km in the southern half of Iceland and falls to about 25 km N from Kverkfjöll; it also falls off to some extent in the coastal belt of south-western Iceland.

I postulate that the spreading rate is more or less proportional to the aggregate width of the active zones, reaching a maximum in the southern half of Iceland, where it is roughly three or four times greater than that in the northern half (which, in turn, is presumably similar to the rate in submerged parts of the Mid-Atlantic Ridge). This is supported by several lines of evidence. First, the total width of outcrop of rocks younger than 3 Myr (formed since

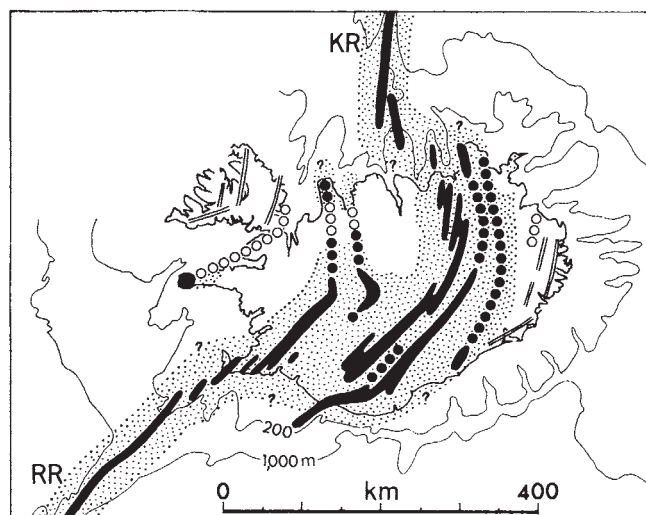


Fig. 3 Summary map of the distribution of rift zones in Iceland and their relationship to the spreading axis along the Mid-Atlantic Ridge axis. KR, Kolbeinsey Ridge¹⁷; RR, Reykjanes Ridge^{14,18}; solid areas, active rift zones; ●, dormant rift zones; ○, extinct rift zones; stippling, crust less than 3 Myr old; —, Tertiary dyke trends. The active zones tend to be parallel with prevalent dyke trends in the Tertiary basalts of eastern¹² and north-western Iceland¹⁹.

the Mammoth Event of the Gauss geomagnetic epoch) reaches a maximum of 250 km in southern Iceland⁹ compared with 140 km in northern Iceland (Fig. 2b) and 60 km in the submerged part of the Mid-Atlantic Ridge⁶ (Fig. 3). The outcrop width on land is admittedly inflated by the overlap of younger lavas on to older lavas, but overlap cannot account for the gross difference in width within Iceland. Second, the frequency of volcanic eruptions (those of the past 1,000 yr are included, Fig. 2c)⁷ for strips of equal width trending in the spreading direction, is several times greater in southern Iceland than elsewhere; the number is taken to be a direct function of the spreading rate. Third, the number of active and dormant silicic volcanic centres is greater in southern Iceland, and by far the biggest centre (Torfajökull) occurs in the south (Fig. 2d); the number and size is taken to be a direct function of the intensity of basaltic magmatism⁸. Finally, the number of active high temperature geothermal fields is greater in southern Iceland than elsewhere (Fig. 2e); it is taken to be a direct function of the intensity of magmatism.

The total discharge pattern of volcanic rock in postglacial time, previously regarded as reaching a maximum in middle Iceland⁹, in fact conforms more closely to the southern Iceland maximum.

Adjustment to varied spreading rates occurs by faulting along transverse fracture zones¹⁰. The two most seismic zones are those between Thingvellir and Katla and west of Manareyjar, with the related Tjornes Fracture Zone¹¹ on land. The scattered postglacial eruptive vents, developed along fissures transverse to the normal fissure trend between Snæfellsjökull and Bifrost, are thought to be related to such adjustments¹⁰, as is the single transverse eruptive fissure east of Hofsjökull.

The locus of maximum spreading is at present situated in the southern half of Iceland. It has been suggested¹² that 10–15 Myr ago the locus was situated farther north where it gave rise to the eastern Iceland Tertiary volcanic pile with its numerous silicic volcanic centres and fossil, high temperature, geothermal fields; it was situated further south 5–10 Myr ago, when the south-eastern Iceland lava pile accumulated with its silicic centres, fossil geothermal fields, and intrusions of gabbro and granophyre; and it has since migrated further

south still. The intensity of volcanism at the locus may also have increased with time.

All of the active zones were active in late moberg times except the part of the Grimsvotn Zone around Katla and Lakagigar, which seems to have become active in the past 10–100 kyr, and the northern sections of several zones were active in late moberg times and have become dormant in the past 10–100 kyr. This suggests that the active zones are transitory and some may have a life of only 1 Myr.

The 20 km strip between Torfajökull and Katla is a dormant zone of young moberg mountains which raises the interesting possibility that the Grimsvotn Zone has moved eastwards, perhaps after being 'spawned off' from the Torfajökull Zone. The double sections of the western zone at Hekla and Myvatn could even be places where 'spawning' is in progress, as could the Thingvellir–Grimsvotn pair. These double zones are, however, more analogous to the pair of parallel dyke swarms, 5 km apart, of the Breiddalur and Thingmuli volcanic centres in eastern Iceland¹³. The period of formation of these centres and of their dyke swarms overlapped, and although subsidence affected the core of each centre no true anticline developed between them. In contrast, an anticline does develop between separate rift zones, as between Thingvellir and Hekla. The difference is, however, one of degree rather than of kind.

The line of the median rift of the Mid-Atlantic Ridge (KR–RR, Fig. 3) has an inflection at Iceland. Where it crosses Iceland this line is dormant or extinct along part of its length and active spreading occurs in part from rift zones lying farther east. One can speculate that new rift zones are generated from time to time, mostly on the convex side of the inflection where expansion can proceed more readily, and most of the excess spreading in Iceland is accommodated by the creation of new crust on that side. The Tertiary basalts of eastern, Iceland may have been rotated and moved northwards by the subsequent creation of excess crust in the southern half of Iceland so that they are now no longer directly opposite the outcrops of similar age in western Iceland.

The prevalent view that the Iceland fissures are purely dilatational and that the magma plays a passive role and rises along them "by invitation" has been questioned¹⁴, and a compressional regime has been indicated by borehole strainmeter measurements¹⁵ and by an analysis of the 1973 Heimay fissures¹⁶. My view that the spreading rate is several times greater in part of Iceland than elsewhere on the Mid-Atlantic Ridge implies that magma plays a very active role in creating and jacking open the fissures there and, indeed, perhaps in causing crustal spreading to occur.

It has often been thought that Iceland offers a unique opportunity to study the details of the spreading process as it operates underwater in a mid-oceanic setting. Iceland is, however, anomalous in that it stands above sea level. I postulate that it is also anomalous for two additional reasons: it has six active rift zones in place of the one believed to exist in submerged sections, and it has a spreading rate which in southern Iceland is several times greater than on submerged parts of the ridge. The question of whether these additional anomalies stem from the fact that Iceland stands above water, or whether Iceland stands above water because of these anomalies, is left open.

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- 1 Saemundsson, K., *Bull. geol. Soc. Am.*, **85**, 494–504 (1974).
- 2 Ward, P. L., *Bull. geol. Soc. Am.*, **82**, 2991–3012 (1971).
- 3 Walker, G. P. L., and Blake, D. H., *Q. Jl. geol. Soc. Lond.*, **122**, 45–61 (1966).
- 4 Piper, J. D. A., *Can. J. Earth Sci.*, **10**, 164–179 (1973).
- 5 Palmason, G., and Saemundsson, K., *A. Rev. Earth Planet. Sci.*, **2**, 25–50 (1974).
- 6 Heirtzler, J. R., Dickson, G. O., Herron, E. M., Pitman, W. C., and le Pichon, X., *J. geophys. Res.*, **73**, 2119–2136 (1968).
- 7 Thorarinsson, S., *Naturufraedningurinn*, **35**, 49–74 (1965).

- ⁸ Walker, G. P. L., in *Geodynamics of Iceland and the North Atlantic area* (edit. by Kristjánsson, L.), 189–201 (Reidel, Dordrecht, 1974).
⁹ Jakobsson, S. P., *Lithos*, **5**, 365–386 (1972).
¹⁰ Sigurdsson, H., *Earth planet. Sci. Lett.*, **10**, 129–135 (1970).
¹¹ Ward, P. L., Palmason, G., and Drake, C., *J. geophys. Res.*, **74**, 665–684 (1969).
¹² Walker, G. P. L., in *Geodynamics of Iceland and the North Atlantic area* (edit. by Kristjánsson, L.), 177–188 (Reidel, Dordrecht, 1974).
¹³ Walker, G. P. L., *Q. J. geol. Soc. Lond.*, **119**, 29–60 (1963).
¹⁴ Einarsson, T., *J. geophys. Res.*, **73**, 7561–7576 (1968).
¹⁵ Hast, N., *Tectonophysics*, **8**, 169–211 (1969).
¹⁶ Brander, J., and Wadge, G., *Nature*, **244**, 496–498 (1973).
¹⁷ Johnston, G. L., in *Geodynamics of Iceland and the North Atlantic area* (edit. by Kristjánsson, L.), 49–62 (Reidel, Dordrecht 1974).
¹⁸ Serson, P. H., Hannaford, W., and Haines, G. V., *Science*, **162**, 355–357 (1968).
¹⁹ Sigurdsson, H., *Rit. vísindafélags íslendinga*, **38**, 162–179 (1967).

Significance of calcium-rich differentiates in chondritic meteorites

KNOWLEDGE of conditions in the early Solar System is based largely on results from the study of meteorites. We have found in two chondritic meteorites 'chondrules' or inclusions containing material with the composition of a calcic plagioclase. We believe that this may be evidence of differentiation on a planetary body before compaction of the chondritic parent body(ies) occurred (see also ref. 1).

The Bovedy Meteorite fell in Northern Ireland in 1969 (refs 2 and 3) and is an unequilibrated ordinary chondrite of the low iron group (L3 in the classification of Van Schmus and Wood⁴). Microprobe analysis of the meteorite showed that one large chondrule, 4 mm in diameter, comprises phenocrysts of low calcium-pyroxene (Fs_{17-28}) with included grains of olivine (Fa_{24}) and a clear isotropic interstitial phase with the composition of a plagioclase (An_{85}). Part of a large bleb, approximately 2 mm across, occurring on another specimen of the meteorite also has the composition of plagioclase (An_{83-88}): an X-ray powder photograph showed no plagioclase lines, indicating that this bleb is mainly glass. It does, however, contain minor whitlockite and an opaque mineral, probably chromite, though these did not show up on the X-ray photograph but were observed during the microprobe work. A 2.55-mg sample of this bleb was analysed for the rare earth elements by an instrumental neutron activation technique⁵ and was found to have a remarkably high, positive europium anomaly. The low abundances of rare earth elements and the magnitude of the europium anomaly are similar to those observed in lunar anorthosites^{6,7} but differ from those of the plagioclases in both the eucritic meteorites^{8,9} and the lunar mare basalts¹⁰ (see Table 1).

The second chondritic meteorite, Goodland, was found in Kansas in 1923 and is classified in the same low iron group as Bovedy but it is petrographically a type 4⁴ and is almost equilibrated¹¹. A chondrule in this stone contains some slightly birefringent material with the composition of a plagioclase (An_{85}) interstitial to crystals of olivine (Fa_{28}) enclosed in orthopyroxene (Fs_{16}).

By analogy with the lunar surface, the calcic plagioclase material of Bovedy probably formed by igneous differentiation on a planetary scale. The same could be true of the similar material in Goodland but the absence of data on the rare earth

elements makes confirmation of this impossible. Accepting this, it is likely that at least one planetary body had been differentiated and partially fragmented so that its disrupted material was incorporated into an L-group, chondritic, parent body. Goodland has a short gas retention age which precludes speculation about its early history¹²; the age of Bovedy is currently under investigation in the hope that it will provide a younger limit to the time of differentiation of the parent of its calcic feldspar glasses.

There seem to have been two distinct periods of heating among planetary bodies in the early Solar System. The first caused the melting and differentiation which led to the production of calcic plagioclase on a planetary body. Portions of this were subsequently available for incorporation into the chondritic parent body which was at this time still relatively undifferentiated. The second heating event caused the metamorphism^{4,11} of at least one (L-group) ordinary chondrite parent body. Differences in the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios between the metamorphosed (H6) chondrite Guarena and the eucrites are also indicative of the occurrence of two heating events separated in time¹³.

Four general sources of energy have been proposed to account for these heating events, namely: the release of accretional energy, the decay of short lived radionuclides, the decay of long lived radionuclides, and intense solar radiation¹⁴. At least two of these are required to account for the two heating cycles outlined above, as each could have been effective once only. Whichever combination of heat sources was responsible for these heating events, evidence for the earlier should best be seen in the least metamorphosed chondrites. It is in these meteorites that the products of the earlier planetary differentiation should be best preserved. We suggest that this preservation has occurred and can be seen in the calcium-rich glass in the Bovedy Meteorite and also, though less certainly, in the calcium-rich material of the chondrite Goodland.

We thank D. Y. Jérôme and J. C. Philippot for the neutron activation analysis, and I. G. Meighan for providing us with the Bovedy sample in which he pointed out the glassy bleb (now BM 1972,233).

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- Hutchison, R. *Nature phys. Sci.*, **240**, 58 (1972).
- Meighan, I. G., and Doughty, P. S., *Nature*, **223**, 24 (1969).
- Andrews, A. D., Rackham, T. W., and Wayman, P. A., *Nature*, **225**, 727 (1969).
- Van Schmus, W. R., and Wood, J. A., *Geochim. cosmochim. Acta*, **31**, 747 (1967).
- Jérôme, D. Y., and Philippot, J. C., *Geochim. cosmochim. Acta*, **37**, 909 (1973).
- Nakamura, N., Masuda, A., Tanaka, T., and Kurasawa, H., *Proc. fourth Lunar Sci. Conf., Geochim. cosmochim. Acta Suppl.*, **4**, 1407 (1973).
- Philippot, J. A., et al., *ibid.*, **4**, 1427 (1973).
- Schnetzler, C. C., and Philippot, J. A., in *Meteorite Research* (edit. by Millman, P. M.), (Reidel, Dordrecht, 1969).
- Mason, B., and Graham, A. L., *Smithsonian Contrib. Earth Sci.*, **3**, 1 (1970).
- Schnetzler, C. C., and Philippot, J. A., *Proc. second Lunar Sci. Conf., Geochim. cosmochim. Acta Suppl.*, **2**, 1101 (1971).
- Dodd, R. T., Van Schmus, W. R., and Koffman, D. M., *Geochim. cosmochim. Acta*, **31**, 921 (1967).
- Heymann, D., *Icarus*, **6**, 189 (1967).
- Wasserburg, G. J., Papanastassiou, D. A., and Sanz, H. G., *Earth planet. Sci. Lett.*, **7**, 33 (1969).
- Wasson, J. T., *Meteorites*, 181–205 (Springer, Berlin, 1974).

Table 1 Rare earth element concentrations (p.p.m.) in Bovedy glass compared with those of lunar and meteoritic samples⁶⁻¹⁰

	Bovedy glass	Lunar anorthosites, bulk data (average of 10)	Plagioclases from lunar mare basalts (average of 12)	Plagioclases from eucrites (average of 3)
Lanthanum	0.25	0.26	7.2	—
Samarium	0.12	0.084	2.4	0.55
Europium	1.35	0.95	3.2	1.1
Ytterbium	0.06	0.051	1.6	0.23
Lutetium	0.0068	0.0085	0.22	—
Europium anomaly	+33	+31	+3.6	+6.0

Pyrite-haematite alteration as a source of colour in red beds and regolith

THE colour in red beds and red soils is generally acknowledged to be caused by the presence of finely dispersed haematite or hydro-haematite. The origin of the haematite remains, however, in dispute¹⁻³. In the case of present-day red weathering in the tropics and subtropics, the haematite or other colouring

material is obviously derived by *in situ* alteration of iron-bearing minerals in the parent rock or soil, but opinion is divided as to whether the haematite or its precursor are of detrital or authigenic origin^{2,3} and Chukhrov¹ considers that red beds could never have formed from laterite. The minerals thought to have altered to give iron oxides and hydroxides are silicates, particularly olivine, pyroxene, biotite and hornblende, and the oxides magnetite and ilmenite. I describe here a case of alteration of pyrite to haematite with the subsequent development of red regolith, and suggest that the alteration of detrital or biogenic pyrite may have more general application to the problem of red beds.

Red-weathered regolith (Munsell colours 5-10R 4-6/5-10) is widespread in the Wellington area of New Zealand⁵. It is considered to have formed in the Pleistocene, during warm interglacial periods when the average annual temperature was at least 3 °C higher than at present, and there was a pronounced dry season. At least three periods of red-weathering are represented in the Wellington area. Near Upper Hutt, 40 km north-east of Wellington, red-weathered horizons underlie and overlie the brown-weathered, rhyolitic Mangaroa Ash⁶. This ash has been dated as mid to late Pleistocene (M. T. Te Punga, personal communication).

During examination of the heavy mineral content of the Mangaroa Ash, I noted numerous small cubes and spherules of haematite. The perfect geometry of the cubes suggested that the haematite was pseudomorphous, and electron probe microanalysis of some of the hardest grains showed them to have a core of iron sulphide of composition corresponding to pyrite or marcasite. In contrast to the almost complete alteration of the pyrite, magnetite and the scarce ferromagnesian silicates were almost completely unaltered. The brown colour of the ash derived from rather weathered brown volcanic glass which was the main constituent and the haematite pseudomorphs did not contribute much to the general colour. In the overlying red-weathered, loess-like material, however, cubes and spherules of haematite occur in all stages of disintegration, and finely dispersed haematite gives the deposit a bright, brick-red colour. Magnetite and ferromagnesian silicates in the loess are relatively unaltered.

Some indication of the conditions in which pyrite alters to haematite is given by the recent or present day alteration of pyrite concretions in Israel⁷, and in Northland, New Zealand. The Israeli concretions are found in an Upper Campanian to Palaeocene calcareous sequence in a particularly arid area of the Negev. Alteration has produced haematite and goethite on the outer parts of the concretions, whereas the inner parts contain jarosite, gypsum, baryte, celestine and sideronatrite. The alteration of the concretions is thought to be very recent, and the preservation of sideronatrite is considered to be a result of the aridity in the area. The fact that the iron oxides and hydroxides are restricted to the outer parts of the concretions is not surprising: phase diagrams⁸ indicate that iron oxides form with increasing pH. In this case the formation of iron oxide is probably influenced by the enclosing calcareous beds. The mean annual temperature in the Negev is about 20 °C. In Northland, New Zealand, pyrite nodules on the beach at Whangaroa are altered to haematite on some surfaces. Whangaroa lies at latitude 35°S, the mean annual temperature is 15.5 °C, and the rainfall is 1,780 mm per annum with a dry season in summer, when temperatures may reach 30 °C. Small areas of laterite have formed on volcanic deposits in the area. In contrast, Upper Hutt has a mean annual temperature of 12.5 °C, and a rainfall of 1,270 mm evenly distributed throughout the year. Haematite is not forming in soils of the Wellington region at present, and the red-weathering is considered to be relict⁵.

The presence of spherules of haematite suggests that biogenic pyrite has been altered in both the ash and overlying loess. I have noted similar pseudomorphs, both cubes and spherules, from Mangaroa Ash at other localities in the Wellington area, and in view of the widespread occurrence of pyrite in sedi-

mentary rocks, pyrite-haematite alteration may be a source of red colour in beds elsewhere. It is perhaps significant that many red beds and red palaeosols are found in environments where pyrite may be expected to be abundant, such as coal measures, lagoons and shallow inland seas². But although magnetite is frequently reported from red beds formed in these environments, I can find few records of pyrite. This suggests that the pyrite may have been altered; the instability of pyrite in oxidising conditions is extremely well known.

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- ¹ Chukhrov, F. V., *Chem. Geol.*, **12**, 67-75 (1973).
- ² Van Houten, F. B., in *Problems in Palaeoclimatology* (edit. by Nairn, A. E. M.), 647-659 (Interscience, New York, 1964).
- ³ Walker, T. R., *Bull. geol. Soc. Am.*, **78**, 353-68 (1967).
- ⁴ Segalen, P., in *Soils and tropical weathering*, 25-38 (UNESCO, Paris, 1971).
- ⁵ Te Punga, M. T., *N.Z. J. Geol. Geophys.*, **7**, 314-39 (1964).
- ⁶ Te Punga, M. T., *N.Z. J. Geol. Geophys.*, **6**, 155-59 (1963).
- ⁷ Sass, E., Nathan, Y., and Nissenbaum, A., *Mineralog. Mag.*, **35**, 84-87 (1965).
- ⁸ Garrels, R. M., *Mineral Equilibria*, 154 (Harper New York, 1960).

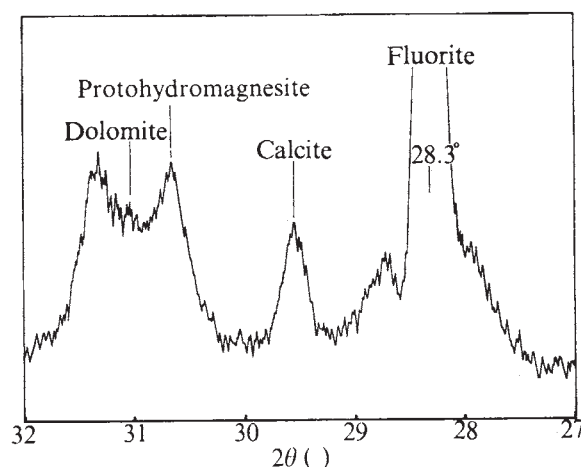
Dolomite and organic material

GEBELEIN and Hoffman¹ drew attention to the association of dolomite with stromatolitic sequences, and suggested that the precipitation of dolomite is related to the presence of organic material. Their experiments confirmed previous results^{2,3} that large quantities of magnesium may be absorbed from seawater by organic material. Magnesium-rich algal filaments may form sites for the precipitation of high magnesium calcite with 17-20 mol % MgCO₃ and although dolomite has not been precipitated in such conditions, it is possible that high-Mg calcite could be diagenetically altered to dolomite because of the high Mg-Ca ratio associated with the organic material¹.

We report here on experimental and field studies, and consider the experimental data of Gebelein and Hoffman in conjunction with other data from localities at which dolomite is precipitated.

In a simulation experiment performed in a large tank⁴, a shallow water, organically rich, evaporitic carbonate environment was simulated and allowed to evolve over a period of eight to nine months. Mineralogical, chemical, and biological parameters of the supernatant and sediment environment were

Fig. 1 X-ray diffraction diagram of sample of decaying filamentous algae sandwiched between calcite and nesquehonite layers.



monitored regularly. An important feature of this experiment was that a layer of decaying filamentous algae was placed between layers of crushed calcite and nesquehonite. At the end of nine months, samples of the decaying filamentous algae were collected, washed, dried, and X rayed (Fig. 1). Dolomite had precipitated within the organic material. This established for the first time that a causal relationship exists between dolomite and organic material. Lippman⁵ showed that dolomite did not precipitate from purely inorganic mixtures of calcite and nesquehonite, a fact verified within our laboratory. The X-ray diagram (Fig. 1) shows in addition that protohydromagnesite⁶, and possibly huntite, also precipitated within the organic material.

One of us (P. J. D.) has studied One Tree Reef, in the southern Great Barrier Reef. In the middle of the rubble cay at the southern end of the reef is a small semipermanent pool, in which carbonates are being precipitated. The sedimentary section within the pool is shown in Fig. 2. An algal mat (Fig. 2a) approximately 3–5 mm thick covers the surface, except near the edge of the pool where land snails are grazing. Below the algal mat is a spongy pale pink to brown organic layer (Fig. 2b) which may be up to 75–100 mm thick. This rests on a white precipitate of aragonite and high magnesium calcite (Fig. 2c). Samples of the organic material were vacuum dried and X rayed (Fig. 2). Dolomite occurs within the organic material, which corroborates the laboratory experiments. It is pertinent also that no dolomite is found in any of the carbonates below the organic layer, and that magnesium increases away from the organic layer (Fig. 2). Furthermore, the dolomite is not present in great quantities, and is not ordered. The conclusion that organic material has formed a site for dolomite precipitation is, however, inescapable.

Although these experimental and field data seem to bear out the convictions of Gebelein and Hoffman¹ that dolomite formation is related to organic material we can suggest a different chemical mechanism from that suggested¹ by those authors.

Their data show that no precipitation occurred unless $(\text{NH}_4)_2\text{CO}_3$ was present. Therefore, the presence of magnesium alone on organic material is of little consequence, irrespective of the Mg–Ca ratios. During their experiment¹, no organic decomposition occurred. Magnesium was therefore not made available for precipitation by organic decomposition, but was scavenged from its organic site by the introduction of $(\text{NH}_4)_2\text{CO}_3$. Our second conclusion is therefore that the catalytic force in the crystallisation of 17–20 mol % magnesium carbonates is the increase in alkalinity. The concentrations used by Gebelein and Hoffman¹ were sufficient to increase markedly the alkalinity of the sample solutions. That this was meant to simulate organic decay is irrelevant. Organic decay did not occur. The data provide an insight into the true relationship between magnesium, alkalinity and the crystallisation of magnesium carbonates.

In most field studies the role of the Mg–Ca ratio has been stressed as critical to the formation of dolomite. Little is ever said of the CO_3 concentration. We believe that conditions for crystal precipitation depend on two parts of a solubility product. The importance of the Mg–Ca ratio is not, however, surprising when it is realised that, with only one exception, no published alkalinity data exist for dolomite precipitating environments. Lippman⁵ has also warned that too much importance has been attached to studies of the Mg–Ca ratio. The solubility product of dolomite is exceeded in seawater^{7–9} but dolomite does not form. Is it also coincidental that the carbonate concentration of seawater is low?

The three modern dolomitic environments which have received most treatment are Bonaire, in the Netherlands Antilles; the Coorong, in South Australia; and the Persian Gulf. Bonaire is the only major locality from which alkalinity data are available. Deffeyes *et al.*¹⁰ report that the alkalinity is markedly higher than in seawater but, more importantly, the CO_3 concentration is greater than the HCO_3 concentration.

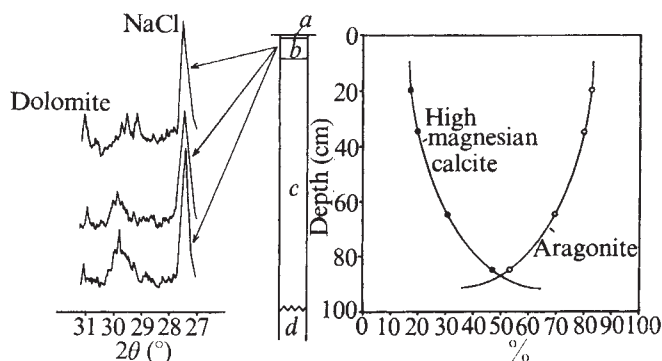


Fig. 2 Sedimentary section within the One Tree Island Pool. a, Algal mat; b, spongy pale pink to brown organic layer, c, precipitated high-magnesium calcite and aragonite; d, coral rubble. X-ray diffraction patterns of the spongy pale pink to brown organic layer show small amounts of carbonate with a dolomitic composition.

No alkalinity data have been published for the Persian Gulf localities, although it is known that salinities are high and the pH is low. The combination of these two factors results in the precipitation of CaSO_4 —which leads to an increase in the solubility of previously precipitated metastable phases—and in the dissolution of these phases with a resulting increase in the alkalinity. Furthermore, a high CO_3 – HCO_3 ratio is suggested by the high salinities.

Von Der Borch¹¹ does not record the alkalinity in the Coorong, mentioning only bicarbonate rich pore waters associated with the hydromagnesite lake. Data from Lake Fellmongery¹² show, however, alkalinity values of up to 5–6 times that of seawater; and Taylor¹² has reported Mg–calcites with 33 mol % MgCO_3 precipitating within the lake. Unpublished data obtained by officers of the Bureau of Mineral Resources show dolomite forming around the eastern shores of Lake Frome in South Australia: a site of carbonate precipitation that coincides with a locality at which groundwater alkalinites are high (eight times that of seawater).

We conclude, therefore, that high alkalinity is extremely important in the formation of dolomite. Organic material may play three different roles: first, living organic material, by virtue of photosynthesis, raises the pH and therefore raises the CO_3 – HCO_3 ratio; second, living organic material can accumulate large quantities of magnesium ion^{1–3}; and third, decaying organic material releases methane which may be used to reduce gypsum, with the concomitant production of CO_2 . Similarly bacterial decay of organic material also produces CO_2 . In both cases, the production of CO_2 will lead to an eventual increase in alkalinity. Various types of organic material, particularly some molecular fractions of humic acids inhibit the precipitation of aragonite. This inhibition may provide sufficient time to nullify the kinetic barriers of dolomite formation.

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¹ Gebelein, C. D., and Hoffman, P., *J. sedim. Petrol.*, **43**, 603–613 (1973).
² Greenfield, L. J., *Ann. N. Y. Acad. Sci.* **109**, 23–45 (1973).

- ³ Berner, R. A., *Science*, **159**, 195–197 (1968).
- ⁴ Bubela, B., and Ferguson, J., *J. sedim. Petrol.*, **43** (8), 1167–1170 (1973).
- ⁵ Lippman, F., *Sedimentary carbonate minerals* (Springer, Berlin, 1973).
- ⁶ Davies, P. J., and Bubela, B., *Chem. Geol.*, **12**, 289–300 (1973).
- ⁷ Garrels, R. M., and Thompson, M. E., *Am. J. Sci.*, **260**, 57–66 (1962).
- ⁸ Hsu, K. J., in *Carbonate Rocks*, vol. 9B (edit. by Chilingar, C. U., et al.), 169–191 (Elsevier, Amsterdam, 1967).
- ⁹ Horne, R. A., *Marine Chemistry* (Wiley-Interscience, New York, 1969).
- ¹⁰ Deffeyes, K. S., Lucia, F. J., and Weyl, P. K., *Soc. Econ. Pal. Min. Spec. Pub.*, **13**, 71–88 (1965).
- ¹¹ Von Der Borch, C. C., *Geochim. cosmochim. Acta*, **29**, 781–799 (1965).
- ¹² Taylor, G. F., *Am. Miner.* (in the press).

Thermal stability of silicon carbide fibres

THE potential of high strength silicon carbide fibres for use in composite materials¹ depends on their thermal stability; specifically on the ability to retain their strength after exposure to the temperatures of composite fabrication and subsequent service. Aveston² has reported a marked reduction in the strength of such fibres after they have been exposed to high temperatures. This has not been observed in single crystal and bulk polycrystalline silicon carbide which exhibits no degradation of mechanical strength with temperature^{3,4}. We have studied the influence of high temperature on the tensile fracture strengths of pyrolytically deposited silicon carbide fibres from several sources. We found that their strengths after exposure were considerably higher than may have been expected from the previously published data.

The four pyrolytically deposited silicon carbide fibres which we examined are listed in Table 1 (fibres A–D). The silicon carbide densities of all of the fibres were less than the theoretical values and the fibre coatings had β silicon carbide structures with varying amounts of a disordered silicon carbide phase. The fibres were heated in air or high purity argon at various temperatures above 1,000 °C. Fracture strengths were measured at room temperature in tension using a gauge length of 25.4 mm. Because the fibres shattered on failure when they were tested in air, all of the tests were performed in an organic liquid (polypropylene glycol 2025) which was sufficiently viscous to prevent shattering but had no significant effect on the fibre fracture strengths. For example, the mean strength of fibre A from six tensile tests in air was $970 \pm 100 \text{ MN m}^{-2}$ compared with a mean strength of $970 \pm 90 \text{ MN m}^{-2}$ for the same number of tests conducted in polypropylene glycol. This procedure permitted the identification and elimination of results from specimens which failed outside the gauge length because of poor alignment, and preserved primary fracture faces for subsequent examination by scanning electron microscopy (SEM).

Table 1 compares the initial strengths of the fibres with their strengths after heating for 48 h at 1,200 °C in air and argon, respectively. The data can also be compared with those obtained by Aveston², although our fibres were present in the hot zone of the furnace throughout the temperature cycle and, consequently, spent about 55 h above 1,000 °C. The mean strengths of our tungsten substrate fibres (that is, fibres A, B and C) after exposure for 48 h at 1,200 °C were found to be between 2.4 and 3.7 times the mean strength of the fibre subjected to similar treatment by Aveston²; the carbon substrate fibre (D) is up to six times stronger, with a mean strength of $1,480 \text{ MN m}^{-2}$ after treatment in air (Table 1).

SEM studies of the fracture faces of fibres A, B and C detected the formation of voids at the original tungsten–silicon carbide interface (Fig. 1a) after treatment at 1,200 °C. The voids could well represent the flaws in the substrate region discussed by Aveston², but we observed that the tungsten in our fibres had reacted to form tungsten silicide phases as well as tungsten carbide. In most cases, however, the flaw controlling the strength was identified as a surface flaw of the type shown

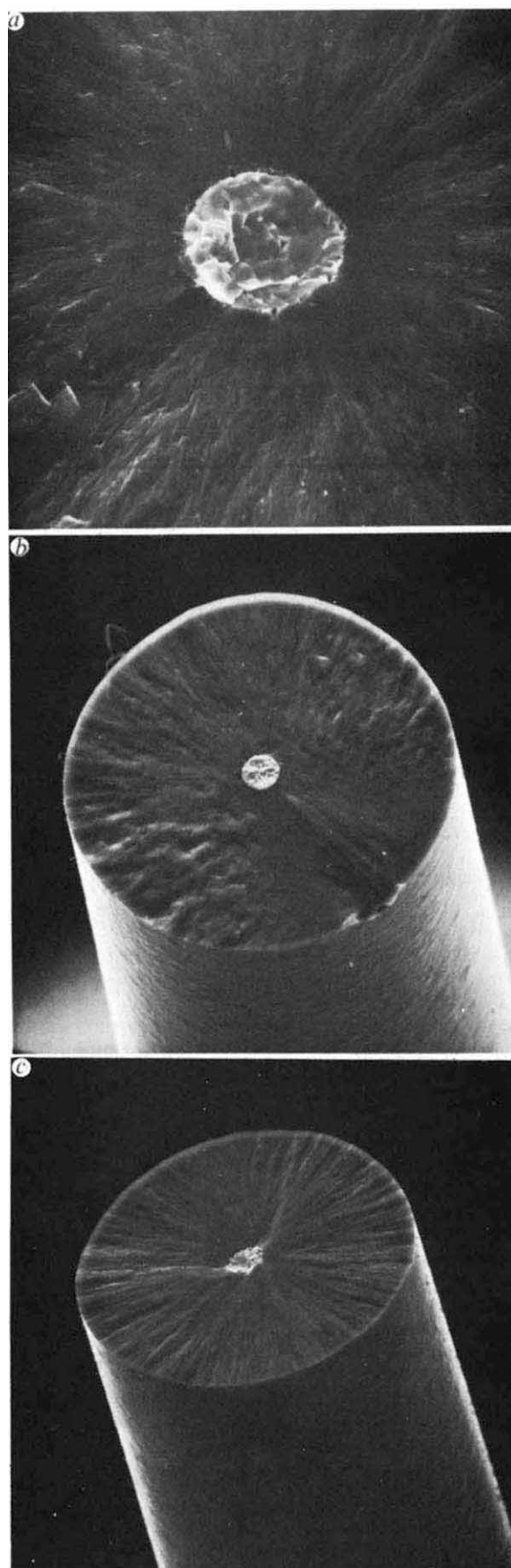


Fig. 1 Fracture surfaces of silicon carbide fibre B heated for 48 h at 1,200 °C. a, Voids at tungsten–silicon carbide interface ($\times 1,680$); b, surface initiated failure ($\times 420$); c, failure probably initiated at tungsten–silicon carbide interface by flaws of type shown in a ($\times 375$).

Table 1 Room temperature fracture strengths of fibres before and after heating in air and argon

Fibre*	Initially		After 48 h at 1,200 °C in air		After 48 h at 1,200 °C in argon	
	Mean strength (MN m ⁻²)	No. tested	Mean strength (MN m ⁻²)	No. tested	Mean strength (MN m ⁻²)	No. tested
A (this work)	970±90	6	700±70	6	600±30	5
B (this work)	2,990±530	12	660±40	8	730±20	5
C (this work)	4,000±140	6	920±130	7	740±90	5
D (this work)	3,990±380	7	1,480±110	6	810±90	5
E (Aveston ³)	2,060		240±30		250±30	

*Fibre A—100 µm diameter with 25 µm tungsten wire substrate; fibre B—100 µm diameter with 12.5 µm tungsten wire substrate; fibre C—150 µm diameter with 12.5 µm tungsten wire substrate; fibre D—100 µm diameter with 33 µm carbon fibre substrate; fibre E—100 µm diameter with 12 µm tungsten wire substrate.

in Fig. 1b. Over 80% of the type A and B fibres treated in air and argon and 50% of the type C fibres heated in argon exhibited fractures initiated at surface flaws. In the case of fibre C heated in air, less than 20% of the failures originated at surface flaws. When surface flaws were not identified as the origin of failure, the critical flaw was difficult to define (Fig. 1c) but the evidence suggests that flaws at the substrate of the type illustrated in Fig. 1a are a likely source of failure.

In the case of the carbon substrate fibre D all failures were attributed to surface flaws after heating in both air and argon, though surface attack was less severe when the fibres were heated in air. As expected, no evidence of reaction between the carbon substrate and the silicon carbide was observed, suggesting that extremely good strength retention might be achieved if surface attack could be avoided.

The important conclusion from our data is that tungsten substrate, silicon carbide fibres available at present retain a useful proportion of their strength after exposure for 48 h at 1,200 °C. Thus, their potential as a high temperature reinforcement in metal or ceramic composites is considerably greater than has been indicated in the past².

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¹ Lindley, M. W., and Godfrey, D. J., *Nature*, **229**, 192–193 (1971).

² Aveston, J., *Nature*, **226**, 146 (1970).

³ Hasselman, D. P. H., and Batha, H. D., *Appl. Phys. Lett.*, **2**, 111 (1963).

⁴ Alliegro, R. A., Coffin, L. B., and Tinklepaugh, J. R., *J. Am. Ceram. Soc.*, **39**, 386 (1956).

¹⁴C carbonate dating and the age of post-Talayotic lime burials in Mallorca

THE study of the cultures of Mallorca in the Balearic Islands, normally recognises pre-Talayotic, Talayotic and post-Talayotic phases. The pre-Talayotic phase covers the interval preceding the adoption of megalithic building techniques. The period of construction of the Talayot towers lasted from about 1500 to 500 BC and was followed by a post-Talayotic phase during which the Talayotic culture underwent a rapid degeneration^{1,2}. In the last centuries of the Talayotic period, Phoenician and Greek influences are evident. Ibiza, about 100 km by sea from Mallorca, was settled by the Phoenicians in 654–653 BC. The Balearics were also involved in wars between Rome and Carthage, between the fifth and second century BC, an involvement which culminated in the conquest of Mallorca in 123 BC by Caecilius Metellus.

Two different definitions of the post-Talayotic phase exist. Garcia² associates the phase with the period following the Roman conquest but Rossello Bordoy¹ gives it as the period during which the true Talayotic culture gave way to a decline that lasted until the 'Romanisation' of the island. We accept the latter definition of post-Talayotic.

During the post-Talayotic, burial customs changed drastically. Normal earth interments were replaced by lime burials, and the use of iron objects among the grave goods became more common³. Lime interments are not found elsewhere in the Mediterranean, and they constitute a strange Mallorcan custom. The origin of the phenomenon is not well understood. Our ¹⁴C dates indicate that the custom originated in the fifth to sixth century BC. Contact with the Phoenicians had already been made by that time, and may have led to the introduction of general lime making techniques. At the beginning of the early Iron Age the Phoenicians in the Canaanite east developed a new method of lining water storage cisterns with impermeable lime plaster for the long term storage of rainwater. Later, a true cement was developed, and many cisterns with such linings exist from the later years of Punic Carthage⁴. The Phoenicians, however, did not use lime burials. Even though the Mallorcan inhabitants may have been familiar with lime making techniques through Phoenician contacts, it is not clear why the technique was adopted for interments.

We examined lime carbonates and incorporated charcoals to determine the feasibility of lime dating for this environment. The majority of the samples we collected, in 1969 and 1973 came from the Son Matge burial site, Mallorca (2°25'E' 39°35'N).

Four charcoal samples from the Talayotic interment phase, taken from directly underneath the lime burial strata, were collected from several excavated sections. The laboratory numbers (QL = Quaternary Isotope Laboratory) and ¹⁴C ages (yr b.p.) of these samples are QL-10, 2,480±70; QL-11, 2,700±80; QL-26, 2,520±80; and QL-27, 2,640±50 respectively. This places the transition from 'regular' burial techniques to lime burial techniques at approximately 2,500 yr b.p. The ¹⁴C age is equivalent in calendar years to 510–660 BC, according to the MASCA calibration tables⁵.

The lime strata in the Son Matge burial grounds have thicknesses of up to 1 m in the central area. A multitude of bone remnants is found in the lime carbonate, but no collagen is left for ¹⁴C dating, because of the high temperatures achieved during lime slaking. Isolated charcoal samples were found in two sections at depths of 75 and 90 cm in the lime layer, close to the contact with the Talayotic burial phase. The ages of these samples (QL-6, 2,520±80 yr b.p. and QL-4, 2,540±110 yr b.p.) confirm that the transition of burial types probably occurred about 2,500 yr b.p.

Charcoal from a shallower section (at 45 cm depth) gave an age of 2,080±90 yr b.p. (QL-8). To this can be added two bone charcoal dates from lime related burials at Son Puig and Muertos Gallard^{2,3} with ages of 2,180±100 and 2,230±80 yr b.p. (Y-1791 and Y-1859, respectively). Evidently, lime burials lasted at least until about 2,100 yr b.p., which is equivalent to a calendar age of 70–110 BC (ref. 5). This post-Talayotic episode could not have lasted much longer, as Roman artefacts occur directly above the lime layer. The curious phenomenon of lime interment was probably practised for about five centuries, from about 600 to 100 BC.

Two pairs of lime carbonate-charcoal samples (QL-8/9 and QL-6/7) were available for a test of the reliability of ¹⁴C dates from the carbonate. The ages of these pairs agree with each

Table 1 Results of Son Matge analyses

Sample no. and location	Depth below surface of burial layer (cm)	$\delta^{13}\text{C}$ (‰)*	^{14}C age (yr b.p.), (corrected for isotope fractionation)
Post-Talayotic lime burial strata			
QL-8 charcoal, section 36	45	-23.0	2,080 ± 90
QL-9 carbonate, section 36	30	-16.8	2,200 ± 100
QL-6 charcoal, section 33	75	-23.4	2,520 ± 80
QL-7 carbonate, section 33	75	-19.9	2,730 ± 100
QL-4 charcoal, section 37	90	-22.5	2,540 ± 110
Y-2669 carbonate, exploratory trench	40	-18.3	2,400 ± 80
Y-2672 carbonate, exploratory trench	80	-13.6	2,240 ± 80
QL-22 carbonate, section 44	20	-11.7	2,260 ± 60
Samples probably associated with Talayotic fire levels			
QL-23 carbonate, section 33	75	-17.7	4,020 ± 50
QL-5 carbonate, section 37	about 90	-19.0 -19.9 -20.9	3,970 ± 100 3,420 ± 100 3,350 ± 60
From Talayotic fire level			
QL-24 'habitational' lime-carbonate	125	-21.4	3,670 ± 70

$$* \delta^{13}\text{C} = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \times 1,000$$

other within the limits of statistical error (Table 1). The ages of three other carbonate samples (Y-2669, Y-2672 and QL-22) also fall in the period of lime interment between 2,500 and 2,100 yr b.p. But two of the carbonate samples (QL-5 and QL-23) are definitely too old and deviate by 900 and 1,500 yr, respectively, from the oldest lime interment ages.

Both carbonate samples with the older ages are found close to the contact zone between the lime burial deposit and the Talayotic fire levels. The lime burials are not always at the same depth, and Talayotic fire levels exist within centimetres of the burial zone. It is possible that the older carbonate was formed after local limestone bedrock was decomposed by overlying Talayotic fires. Sample QL-24 is a clear example of such 'habitational' lime carbonate; its age of $3,670 \pm 70$ yr b.p. is in excellent agreement with the age of the charcoal from associated fires. In our opinion, the two older carbonate samples are associated with Talayotic fire levels, and are not part of the lime burial deposit.

The $\delta^{13}\text{C}$ values listed in Table 1 represent the deviation (‰) of the sample $^{13}\text{C}/^{12}\text{C}$ ratio from the PDB standard. The $\delta^{13}\text{C}$ values of the carbonate samples of 'normal' ^{14}C age average -16.1 ‰. The lime at Son Matge has probably been made from the limestone cave wall which has a $\delta^{13}\text{C}$ value of -1.4 ‰. If the older ages of the two carbonates already mentioned had been caused by incomplete outgassing of the limestone during lime fabrication, the expected $\delta^{13}\text{C}$ values would be higher. Age differences of 900–1,500 yr could be caused by the addition of up to 20% bedrock limestone, resulting in a maximum increase in the $\delta^{13}\text{C}$ ratio of 3‰. But the $\delta^{13}\text{C}$ values of the 'anomalous' samples average -19.4 ‰; 3.3‰ below the -16.1 ‰ found for the carbonate samples with the proper ^{14}C age. This means that incomplete outgassing is unlikely to be the cause of the older carbonate ages.

This analysis indicates that ^{14}C dating of carbonate associated with lime burials, yields reliable ages. Unfortunately the range of $\delta^{13}\text{C}$ values, from -12 to -21 ‰, is largely a result of kinetic effects. Thus, anomalous ages cannot always be detected using

^{13}C ratios. Interment lime dating, on average, yields much better results than mortar dating, but a criterion that always distinguishes normal material from anomalous material is not yet available.

The accurate ^{14}C dating of mortar depends on the extent of contamination by ancient carbon resulting from the use of calcareous sands and/or incompletely kilned limestone⁶⁻⁸. As these factors depend on geographical location, a few Mallorcan samples were measured. Stucco from the Pollentia Roman Republican building at Alcudia, dating from about 30 BC, has a very anomalous age of 8,000 yr b.p. (Y-2668). On the other hand, stucco from the Santa Catalina de Sena excavation, dated (through its association with Moorish pottery) at about 1,000–1,200 AD, has a ^{14}C age of 950 ± 100 yr b.p. (Y-2671). Evidently, the lime making techniques of these two cultures were quite different.

We also investigated the ^{14}C age of lime carbonate at the Son Oms site, formed after the local limestone bedrock was decomposed by overlying fires. There, a completely anomalous age of 20,750 yr was obtained (QL-19), but in that instance the $\delta^{13}\text{C}$ ratio of -5 ‰ clearly showed that incomplete degassing of the original limestone was the cause of the discrepancy.

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- Rossello Bordoy, G., *La Prehistoria de Mallorca* (Palma, 1972).
- Pericot Garcia, L., *The Balearic Islands* (Praeger, New York and Washington, 1973).
- Waldren, W. H., *A Unique Prehistoric Weapon from the Balearic Island of Mallorca* (Deya Archaeological Museum, Deya de Mallorca, 1970).
- Harden, D., *The Phoenicians* (edit. by Daniel, G.), (Thames and Hudson, London, 1963).
- Ralph, E. K., Michael, H. N., and Han, M. C., *Radiocarbon Dates and Reality*, MASCA Newsletter of the Applied Science Center for Archeology (University of Pennsylvania, 1973).
- Baxter, M. S., and Walton, A., *Nature*, 225, 937 (1970).
- Stuiver, M., and Smith, C. S., *Proc. 6th Int. Conf. Radiocarbon and Tritium Dating*, Pullman, Washington, 1965, 338 (US Department of Commerce, 1965).
- Delibrias, G., and Labeyrie, J., *ibid.*, 334.

Mechanical state of elastin

WE describe experiments in which the mechanical state of elastin was determined in conditions comparable to those used by Weis-Fogh and Anderson^{1,2}. In their mechanocalorimetric experiments a strain was imposed on a specimen immersed in diluent in a calorimeter, maintained for several hundred seconds, and then released. Measurements of heat release led them to reject the classical theory of rubber elasticity³⁻⁶ which, for solvated elastin, they replaced by a new model, the liquid-drop elastomer. There is, however, good evidence that in neglecting the differential heat of solution Weis-Fogh and Anderson neglected a dominant source of heat release^{7,8}. Our purpose is to examine the alternative assumption, that elastin conforms to the classical theory of rubber elasticity in the experimental conditions they studied.

A small elastin ring (sliced from pig aorta and purified according to the method of Gotte *et al.*⁹) was mounted in an inverted torsion pendulum¹⁰, immersed in solvent and held in a vertical position hooked over supporting metal wires. The ring was elongated by a very small tensile load so that the specimen formed two parallel lamellae, slightly displaced from one another. Let the dimensions of each lamella at temperatures T_0 and T be a_0 , b_0 , c_0 and a , b , c . Let the specimen be isotropic so that if $a = \gamma_T a_0$ then $b = \gamma_T b_0$ and $c = \gamma_T c_0$ in which γ_T

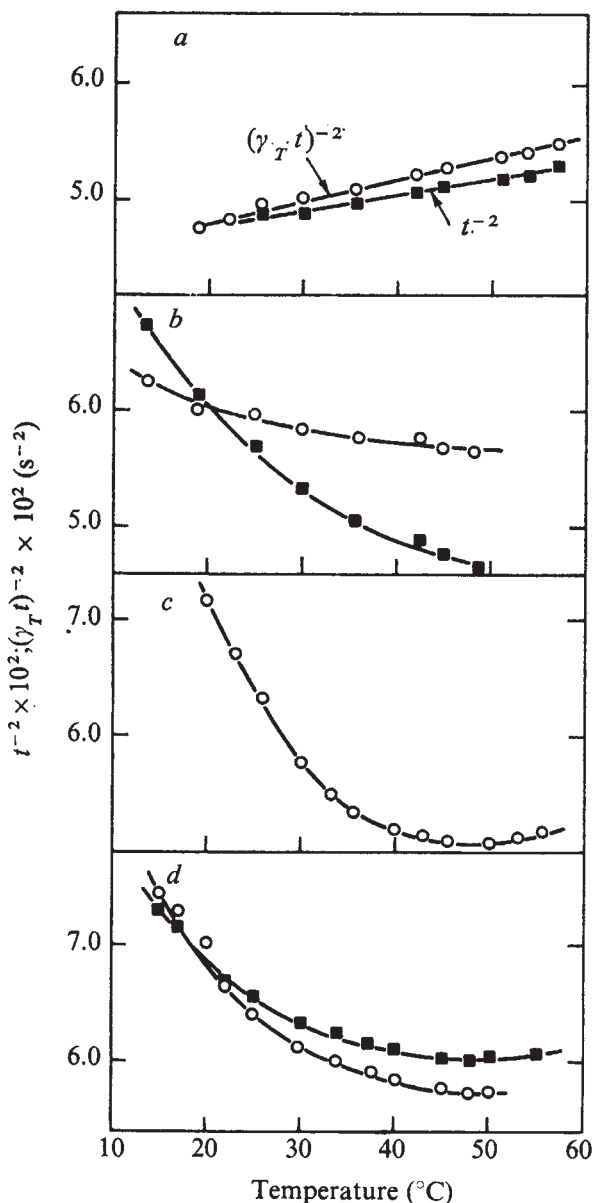


Fig. 1 Temperature dependence of the inverse square of the period of oscillation of the torsion pendulum corrected (○) and uncorrected (■) for change in specimen dimensions as a result of swelling in water-ethanol solutions and formamide. The theory of rubber elasticity predicts the plot of $(\gamma_T t)^{-2}$ as a function of T to be linear and of positive slope (equations (4) and (5)). a, Formamide; b, water; c, water-20% ethanol; d, water-50% ethanol.

is a parameter which depends only on temperature. It follows that if the moment of inertia of the pendulum is constant the ratio of the shear moduli at T and T_0 is¹⁰,

$$G_T/G_{T_0} = (1/\gamma_T^3) (t^{-2}/t_0^{-2}) \quad (1)$$

in which t and t_0 are the observed periods of oscillation of the pendulum at T and T_0 . It was not possible to observe the logarithmic decrement of the specimen because of the viscous drag of the solvent in which the specimen was immersed. The period t (~ 4 s) was determined at different temperatures with a stop watch. The temperature dependence of solvent uptake was obtained by weighing and γ_T was computed assuming the density of the absorbed diluent to be equal to the liquid density.

According to the prediction of the theory of rubber elasticity¹¹,

$$G_T/G_{T_0} = T/T_0 (V_{T_0}/V_T)^{1/3} \langle r^2 \rangle_0^{T_0} / \langle r^2 \rangle_0^T \quad (2)$$

in which V is the specimen volume in equilibrium with the diluent and $\langle r^2 \rangle_0$ is the mean square end-to-end distance of the network chains unrestricted by cross links (at temperatures T and T_0 as indicated). It follows that

$$\ln G/dT = 1/T - (1/3) \beta_V - \beta \langle r^2 \rangle_0 \quad (3)$$

in which β is the temperature coefficient of volume and of $\langle r^2 \rangle_0$ according to subscript. For water at 20 °C and taking account of the errors involved in measuring β_V and $\beta \langle r^2 \rangle_0$ we may neglect $\beta \langle r^2 \rangle_0$ ($= 0.5 \times 10^{-3}$, ref. 6) in comparison with $1/T$ (3.41×10^{-3}) and $\beta_V/3$ ($= -4.5 \times 10^{-3}$, ref. 6). Since the value of $\beta \langle r^2 \rangle_0$ for water seems unusually large (compared with ethylene glycol and dimethyl sulphoxide⁶) and in the absence of evidence to the contrary (see also ref. 7) we take $\beta \langle r^2 \rangle_0$ to be negligible for water-ethanol solutions and for formamide. Therefore rewriting equation (2) with $\langle r^2 \rangle_0^T / \langle r^2 \rangle_0^{T_0} = 1$ and $\gamma_T = (V_T/V_{T_0})^{1/3}$

$$G_T \gamma_T = AT \quad (4)$$

in which the constant $A = (G_{T_0}/T_0)$. From equation (1), the observed value of $G_T \gamma_T$ is

$$G_T \gamma_T = B(\gamma_T t)^{-2} \quad (5)$$

in which B is a constant equal to $G_{T_0} t_0^2$. So a specimen conforming to this rubber elastic model will exhibit a linear dependence between $(\gamma_T t)^{-2}$ and T . If the transition from the glassy to the rubbery state is incomplete and the specimen significantly viscoelastic then a plot of $(\gamma_T t)^{-2}$ against T will not be linear and will be of negative slope^{10,12,13}.

It is not the purpose of these experiments to determine precisely the relative magnitude of f_e and f_s , the energy and entropy components of the mechanical force. The torsion pendulum is not well suited for this task. The purpose is much simpler; briefly stated, it is to determine whether or not the temperature dependence of $G_T \gamma_T$ is negative or positive.

The temperature dependence of t^{-2} and $(\gamma_T t)^{-2}$ is shown in Fig. 1 for elastin immersed in formamide, pure water and water-ethanol solutions containing 20% and 50% ethanol (v/v). The elastin-formamide system shows a linear dependence between $(\gamma_T t)^{-2}$ and T with a positive slope in accordance with the theory of rubber elasticity. The behaviour of elastin in the other solvents is typical of an amorphous polymer at temperatures in the viscoelastic transition zone; the plot of $(\gamma_T t)^{-2}$ as a function of T is a curve of negative slope^{10,12,13}. This important finding was checked using step-function experiments at greater mechanical strains.

An elastin ring was looped over a pair of Invar rollers (held in an Invar jig) and mounted in an Instron testing machine. The elastin ring was immersed in diluent at constant temperature. At the start of a stress relaxation experiment ($t = 0$) the roller separation was changed rapidly (within 1 s) from one predetermined position to another and then maintained at this second position. The roller displacement was calculated to yield a strain in the specimen close to 30%. The relaxation of the force was observed out to $t = 190$ s, the first measurement being taken at $t = 2$ s. This was repeated at temperatures in the range 0–60 °C, the specimen being allowed to relax between experiments. At each temperature the machine moved the rollers automatically through the identical displacement.

The temperature dependence of the force $f(t)$ is shown in Fig. 2 for elastin immersed in 20–80 ethanol-water solution. The interpretation for this system is particularly straightforward since γ_T is close to unity in the temperature region

0–60 °C. The observed behaviour is in complete accord with the torsion pendulum experiment. The temperature dependence of $f(t)$ (negative and curved) and the time dependence (greatest at lowest temperature) is typical of an amorphous polymer in the viscoelastic transition zone.

The relationship of the determined values of $f(t)$ to force-temperature observations obtained in a conventional thermoelastic experiment was made clear in the final experiment. After the final determination of $f(t)$ at 58.4 °C the specimen was maintained at that temperature under constant length for 120 min. The value of the force at this time, designated f_R , is shown in Fig. 2. The temperature was then lowered and measurements of f_R taken at five temperatures between 58.4 and 1.6 °C (Fig. 2). The reversibility of the f_R - T curve was checked by taking two further f_R points at 32.6 and 45.8 °C (Fig. 2). It will be seen that f_R is of positive slope at low temperatures and essentially zero slope above 50 °C.

The usual thermoelastic procedure for studying elastomers is to deform at the highest temperature for some time (for example, overnight⁶) and then to observe the temperature dependence of force at constant length. The resulting force temperature plot is analogous to the f_R - T plot of Fig. 2. But this experiment does not measure the mechanical state in the conditions described by Weis-Fogh and Anderson. The essential difference is one of time: short time (10–1,000 s) in their experiment, and pseudo infinite time in the determination of f_R . For the elastin-formamide system this difference in time scale is not significant: our observations show that the $f(t)$ and f_R plots (as functions of T) are of positive slope and essentially parallel. It follows that for this highly swollen system the theory of rubber elasticity is adequate. But for the less swollen elastin-water and elastin-water/ethanol systems the time dependent force $f(t)$ will depend on other parameters in addition to f_R , notably the distribution of relaxation times for the relaxation process.

It follows that elastin in the vasculature operates under conditions of time, temperature and solvent uptake which place it in the viscoelastic transition zone. The equivalent time in step function experiments may be approximated, $t = (2\pi\nu)^{-1}$ in which ν is the oscillation frequency: for $\nu = 1$ Hz $t = 0.16$ s. Stress relaxation experiments at times down to 2 s on the elastin-water system support the observation shown in Fig. 1

that at body temperature and under the oscillatory conditions of the vasculature elastin will be significantly viscoelastic. The most significant physiological implications of this are mechanical damping and the response of the solid to the hydrostatic component of the stress tensor. As emphasised by Hoeve and Flory⁷ the volume of the swollen specimen increases with length; $(\partial V/\partial L)_{p, T, eq} > 0$ in which eq denotes the specimen is in equilibrium with a reservoir of diluent. When the stress is applied the specimen absorbs liquid which is desorbed when it is released. Studies of non-diluted polymers show this volume change to increase when the specimen is cooled from the rubber elastic into the viscoelastic transition zone^{14,15}. If this is true for diluted elastin then a pulsating stress in an element of elastin will generate a molecular flux along a concentration gradient which may well be considerably larger than the flux due to normal diffusion processes. The efficiency of this stress assisted diffusion will be facilitated by a laminated texture, as in the vasculature.

We note finally the deviation of the f_R - T data from those near in form to proportionality between equilibrium force and absolute temperature, which is predicted⁵ for a system in which: (i) the internal energy contribution to total retractive force is small ($f_e/f \ll 1$); (ii) disproportionation of solvent within the polymer phase is negligible; (iii) equilibrium swelling is independent of temperature ($\gamma_T = 1$). Our work indicates that although γ_T varies almost imperceptibly with temperature in the 20–80 ethanol-water solution, the temperature dependence has a magnitude large enough to modify the slope of the curve significantly. Furthermore, the importance of (ii) as a contributory factor remains in dispute. We do not find any discrepancy between the form of the f_R - T curve obtained and the claim of near ideal elasticity for swollen elastin in the equilibrium state ($f_e/f \ll 1$). A more precise examination of the implications of experimental data obtained on elastin will require a realistic consideration of the small anisotropy known to be present.

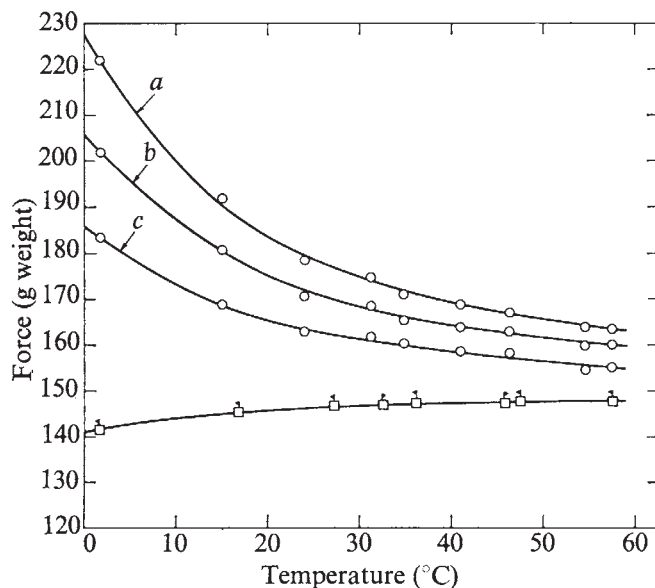
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Fig. 2 Temperature dependence of force at constant length for elastin in 20–80 ethanol-water solution; ○, $f(t)$ for values of t equal to 2 s (a), 10 s (b), 190 s (c); □, f_R . The flag on the f_R points indicates direction of temperature change.



- Weis-Fogh, T. and Anderson, S. O., *Chemistry and Molecular Biology of the Inter-cellular Matrix*, (edit. by Balazs, E. A.), 1, 671 (Academic Press, London 1970).
- Weis-Fogh, T., and Anderson, S. O., *Nature*, **227**, 718 (1970).
- Flory, P. J., *Principles of Polymer Chemistry* (Cornell University Press, Ithaca, 1953).
- Hoeve, C. A. J., and Flory, P. J., *J. Am. chem. Soc.*, **80**, 6523 (1958).
- Hoeve, C. A. J., and Flory, P. J., *J. Polym. Sci.*, **60**, 155 (1962).
- Mistrali, F., Volpin, D., Garibaldi, B. B., and Ciferri, A., *J. phys. Chem.*, **75**, 142 (1971).
- Hoeve, C. A. J., and Flory, P. J., *Biopolymers*, **13**, 677 (1974).
- Grut, W., and McCrum, N. G., *Nature*, **251**, 165 (1974).
- Gotté, L., Stern, P., Elsdén, D. S., and Partridge, S. M., *Biochem. J.*, **87**, 344 (1963).
- McCrum, N. G., Read, B. E., and Williams, G., *Anelastic and Dielectric Effects in Polymeric Solids* (Wiley, London, 1967).
- Smith, K. J., in *Polymer Science* (edit. by Jenkins, A. D.) Ch. 5 (North Holland, Amsterdam, 1972).
- Tobolsky, A. V., *Properties and Structure of Polymers*, (Wiley, New York, 1960).
- Ferry, J. D., *Viscoelastic Properties of Polymers* (Wiley, New York, 1961).
- Theocaris, P. S., and Hadjiioseph, Chr., *Kolloidzeitschrift*, **202**, 133 (1965).
- Theocaris, P. S., *Kolloidzeitschrift*, **235**, 1182 (1969).

Cellulase activity and niche separation in freshwater gastropods

ALTHOUGH there is considerable morphological similarity between different species of freshwater snail in terms of general body form and radular construction¹, differences in diet occur^{2,3}. Furthermore, preferred food materials are invariably digested more efficiently than less preferred materials⁴ (Fig. 1). The implications are that differences in diet and resultant niche separations are dependent on physiological, rather than morphological, divergence and that the former is probably related to the quality and quantity of enzyme secretion. Thus it may be

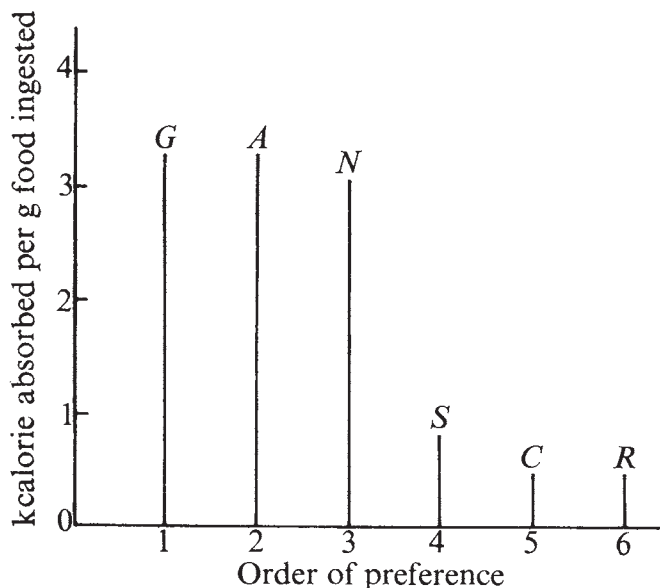


Fig. 1 Nutritive value of food, in terms of potential energy absorbed across the gut wall per weight of food ingested (derived from ref. 4), against the preference order of food materials to *Ancylus fluviatilis*. The latter were obtained from independent laboratory and field observations¹³. Intervals between the preference values are not equal and as yet have not been quantified. Similar results have been obtained for *Planorbis contortus* and *Lymnaea pereger* and suggest that snails prefer those food materials which provide most potential energy per mouthful. This dependency may not be linear. G, *Gomphonema*; A, *Achnanthes*; N, *Navicula*; S, *Scenedesmus*; C, *Cladophora*; R, *Rivularia*.

possible to reconcile the conflict first noted by Boycott⁵ between the general observation that most species of freshwater gastropod can and do coexist^{5,6} and the so-called "competitive exclusion principle". To test this hypothesis we have examined cellulase activity in 14 species of snail and have linked our results to known dietary differences and to direct observations on assimilation efficiencies. We have found a strong correlation between enzyme activity and assimilation efficiency, and that dietary preferences can be ascribed to physiological differences of this kind.

Snails were obtained from Malham Tarn (Yorkshire, UK). Enzymes were extracted from starved snails⁷ and were used immediately. Activity measurements were made by estimating the amount of reducing sugar liberated from type II cellulose⁷. Enzyme extract (4 ml) was added to 50 mg of substrate in 1 ml

of 0.1 M phosphate buffer (pH 5.5) and incubation was for 6 h at 4 °C. These conditions are optimum for the activity of molluscan cellulases⁷. The reaction was stopped by adding 1 ml of 0.1 N NaOH. Blank incubations were made using the enzyme extract without cellulose and cellulose without enzyme. Corrections were subsequently applied to the experimental replicates for the small amount of reducing sugar initially present in each. Protein concentrations of the original extracts were estimated from separate subsamples⁸.

The efficiency with which snails digest green algae was used as an index of their ability to digest and assimilate cellulose. *Scenedesmus* sp. was used as food supply because it has comparatively thick cellulose walls which increase its resistance to herbivorous grazing⁹. Assimilation efficiencies were measured using a 'double isotope' technique involving ¹⁴C and ⁵¹Cr (ref. 10). All snails were starved for 3 d before use and determinations were carried out at 10 °C. It was assumed that each species absorbs about 10% ⁵¹Cr as in *Planorbis contortus* L. and *Ancylus fluviatilis* Müll.¹⁰.

The results of both cellulase activity and assimilation efficiency are given in Table 1. Previous work on cellulase activity in *Lymnaea stagnalis*¹¹ gave values of 272 and 263 units (as defined in Table 1) for the crop-gizzard apparatus and hepatopancreas respectively. The latter are extremely similar to our results. Throughout we have ignored questions concerning the source of cellulases in molluscs^{11,12} (that is, from bacterial or snail tissue) because from a dietary point of view, all that is required is that the enzymes are present.

Gut extracts from different snail species show differences in cellulase activity (Table 1) and these reflect an underlying physiological variability. There is also a positive correlation between cellulase activity and the ability of a particular species to digest *Scenedesmus*. This is illustrated in Fig. 2, but no line is drawn through the points because the theoretical relationship between enzyme activity and digestive efficiency is not known. A simple linear relationship is, however, suggested and a cause-effect association is implied.

The results presented in Table 1 and Fig. 2 conform to the previously reported findings on dietary preferences and to the fact that snails tend to prefer foodstuffs which are easiest to digest (see Fig. 1). Thus *L. pereger obtusa* seeks out, prefers and is able to digest, green algae with thick cellulose walls². It has been suggested that this emphasis on green algae may apply to the lymnaeids in general² and Table 1 shows that these snails do have a high cellulase activity. *A. fluviatilis*, however, has a low cellulase activity and tends to prefer epilithic diatoms without a cellulose wall but which have a tough, protective siliceous shell¹³. The gizzard musculature is better developed

Table 1 Cellulase activity and assimilation efficiency (with respect to *Scenedesmus* spp.) in a variety of freshwater gastropods

Species	Whole organism†	Cellulase activity* Crop-gizzard‡	Hepatopancreas‡	Assimilation efficiency† (%)§
Pulmonata				
<i>Lymnaea stagnalis</i> L.	—	275	270	76.0 (±6.8)
<i>Lymnaea pereger</i> L.	—	246	210	71.5 (±8.6)
<i>Lymnaea truncatula</i> Müll.	256	—	—	70.1 (±5.1)
<i>Lymnaea palustris</i> Müll.	—	229	221	62.4 (±6.1)
<i>Physa fontinalis</i> L.	—	104	96	39.1 (±4.3)
<i>Ancylus fluviatilis</i> Müll.	28	—	—	13.9 (±3.8)
<i>Planorbis planorbis</i> L.	—	151	182	46.9 (±5.1)
<i>Planorbis corneus</i> L.	—	—	243	—
<i>Planorbis albus</i> Müll.	40	—	—	26.0 (±4.8)
<i>Planorbis contortus</i> L.	32	—	—	7.7 (±2.1)
Prosobranchia				
<i>Bithynia tentaculata</i> L.	—	281	284	89.0 (±9.2)
<i>Potamopyrgus jenkinsi</i> Smith	269	—	—	78.0 (±7.3)
<i>Valvata cristata</i> Müll.	340	—	—	92.4 (±10.0)
<i>Valvata piscinalis</i> Müll.	329	—	—	94.7 (±7.8)

*Cellulase activity = $\frac{\mu\text{g reducing sugar per ml incubation mixture}}{\text{mg protein per ml extract}}$

†In terms of carbon¹⁰.

‡Average of 3 determinations; maximum range ±5–7 % mean.

§Average of 20 determinations; confidence limits = $2s/\sqrt{n}$ where s is standard deviation, n is 20.

||Obtained from Stone and Morton¹¹.

in this species¹³, so here the emphasis seems to have shifted from that of digesting the cellulose walls chemically, to that of rupturing the diatom shells mechanically. Cellulase activity in the Planorbidae is more variable. The larger species (for example, *Planorbis planorbis* and *P. corneus*) have an active cellulase and with a predominantly detrophagous habit² may be able to make use of the non-living, organic, detrital fraction (that is, cellulose). The smaller species like *P. contortus*, however, have a low cellulase activity and this may result in (or be the result of) a bacterial feeding mode¹⁴. Little is known about the dietary requirements of the freshwater prosobranchs but as suggested by the high cellulase activity, they do seem to feed on green plants and/or detritus. For example, *Bithynia tentaculata* feeds on water weeds for most of the year but feeds on detrital particles during the winter¹⁵ and one of us (P.C.) has observed some populations of *Potamopyrgus jenkinsi* feeding exclusively on detritus and others feeding on algal epiphytes in which green unicells were dominant (unpublished).

Although the variations discussed are limited to feeding behaviour, this aspect of the niche is believed to be critical in determining the presence, absence and intensity of competition between species¹⁶. Differences in food requirements may also impose subtle differences on microdispersion patterns⁶ which can lead to corresponding spatial separation; from this point of view it is interesting to note that Harman¹⁷ has found a convincing relationship between substrate diversity and species diversity in freshwater snail communities. The problem of coexistence can thus be solved, in a general way, by reference to physiological variations leading to distinctions in the food, and possibly the living space requirements of different taxonomic groups. This, of course, does not completely preclude the possibility of competition and there are examples of replacement and competitive exclusion in snail communities. These, as might be expected, however, involve species with similar enzyme activities and similar dietary requirements; notably the lymnaeids and the prosobranchs. For example, *L. peregere* tends to replace both *L. truncatula* and *L. palustris*, which are typical fugitive species (unpublished), and Harman¹⁸ has recorded competition effects between *Bythinia* and pleuroceriids. In addition, starved snails become less selective in their feeding behaviour⁴ and when faced with algal monocultures in laboratory conditions, they tend to ingest more of the least well

digested food materials⁴. This means that reductions in the supply of preferred food, on either a spatial or temporal scale, can lead to less discriminate feeding and may result in temporary niche overlap.

Our results on cellulase activity support the hypothesis that in freshwater snails, niche separation depends on physiological rather than morphological differences. Snails seem to have reached an adaptive peak morphologically and subsequent niche separation seems to have occurred by physiological divergence. What is more, this may operate at an intraspecific level¹⁹ so that differences in cellulase activity and thus dietary requirements may occur between different, but separated, populations of the same species. Physiological, as opposed to morphological, divergence also seems to be important in the Drosophilidae²⁰, in the Oligochaeta²¹ and probably in the Nematoda. Coexistence therefore becomes possible in snails in spite of the superficial similarity of different species. Where enzyme activity is similar between species there is *prima facie* evidence for competition and this may also occur between more divergent species in conditions of food shortage.

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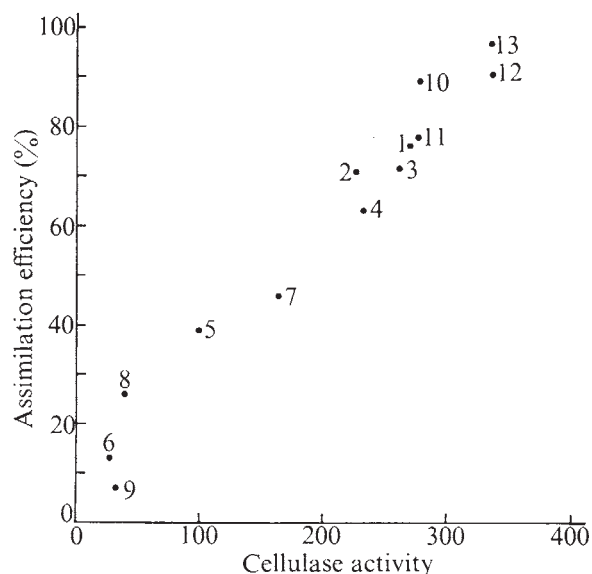
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- ¹ Hyman, L. H., *The Invertebrates*, 6 (McGraw-Hill, New York, 1967).
- ² Calow, P., *Proc. malac. Soc. Lond.*, **39**, 203–215 (1970); **40**, 483–489 (1973).
- ³ Mason, C. in *Biology of Plant Litter Decomposition*, 2 (edit. by Dickinson, C. H., and Pugh, G. J. F.), 555–591, (Academic, New York, 1974).
- ⁴ Calow, P., *Oecologia* (in the press).
- ⁵ Boycott, A. E., *J. Anim. Ecol.*, **5**, 116–186 (1936).
- ⁶ Calow, P., *Freshwater Biol.*, **4**, 1–20, (1974).
- ⁷ Koopmans, J. J. C., *Neth. J. Zool.*, **20**, 445–463 (1970).
- ⁸ Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, **193**, 265–275 (1951).
- ⁹ Round, F. E., *Soc. Sci. Fennica Commentationes Biol.*, **217**, 1–13 (1959).
- ¹⁰ Calow, P., and Fletcher, C. R., *Oecologia*, **9**, 155–170 (1972).
- ¹¹ Stone, B. A., and Morton, J. B., *Proc. malac. Soc. Lond.*, **33**, 127–141 (1958).
- ¹² Crosby, N. D., and Reid, R. G. B., *Can. J. Zool.*, **49**, 617–622 (1971).
- ¹³ Calow, P., *Oecologia*, **13**, 113–133 (1974).
- ¹⁴ Calow, P., *Proc. malac. Soc. Lond.*, **41**, 145–156 (1974).
- ¹⁵ Cleland, D. M., *Proc. malac. Soc. Lond.*, **30**, 167–203 (1954).
- ¹⁶ Weatherley, A. H., *Growth and Ecology of Fish Populations* (Academic, London and New York, 1972).
- ¹⁷ Harman, W. N., *Ecology*, **53**, 271–277 (1972).
- ¹⁸ Harman, W. N., *Nautilus*, **81**, 77–83 (1968); **82**, 72–73 (1968).
- ¹⁹ Russell-Hunter, W. D., *Aquatic Productivity* (Macmillan, New York, 1970).
- ²⁰ Dobzhansky, T., *Genetics of the Evolutionary Process* (Columbia University Press, New York, 1970).
- ²¹ Brinkhurst, R. O., and Chua, K. E., *J. Fish. Res. Bd. Can.*, **26**, 2659–2668 (1969).

Fig. 2 The efficiency with which snails digest *Scenedesmus* spp. against the cellulase activity (defined in Table 1) of their gut extracts. Where the activity of crop-gizzard apparatus and hepatopancreas were determined separately these have been averaged. 1, *Lymnaea stagnalis*; 2, *L. peregere*; 3, *L. truncatula*; 4, *L. palustris*; 5, *Physa fontinalis*; 6, *Ancylus fluviatilis*; 7, *Planorbis planorbis*; 8, *P. albus*; 9, *P. contortus*; 10, *Bithynia tentaculata*; 11, *Potamopyrgus jenkinsi*; 12, *Valvata cristata*; 13, *V. piscinalis*.



Retinal resistance barriers and electrical lateral inhibition

EXTRACELLULAR fields around active neural tissues are usually only a small fraction of the transmembrane voltages generating them, but this is not so in the arthropod compound eye. Light-evoked fields (the electroretinogram, ERG) can range over 10 mV amplitude¹ and grow even larger near the receptor endings at the first synapse, the lamina (Fig. 1). The physical basis for such large fields is not understood although it has been suggested that there may be barriers of high resistance in the extracellular space^{2,3} across which current flow may generate large voltages. Direct measurements of extracellular resistance profiles in the insect eye reported here indicate that a barrier does exist. I also show the effect such a barrier must have on the retinal currents produced by light, which is to set up a system of electrical presynaptic inhibition on certain of the receptor terminals, acting through the extracellular space. When this neural circuit is defined in detail it can be shown that it specifically selects for inhibition only those neighbouring receptors with properties dissimilar to the excited elements. Such a system might be of general interest as a mechanism of inhibitory interaction, since it can act in a graded manner, needs no neural wiring to set it up, and no genetic programme to account for the selectivity its inhibition shows. The developmental problem of how a neurone can establish highly specific connections with follower cells^{4–6} and yet at the

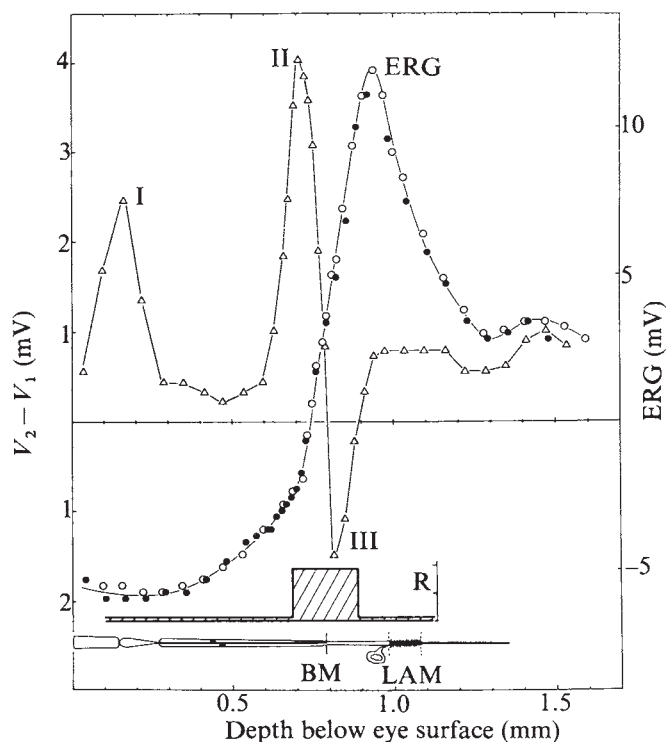


Fig. 1 Simultaneous measurements in locust eye of the depth profiles of ERG peak amplitude ($\circ$, $\bullet$) and apparent relative retinal resistance (Δ). The latter expresses the voltage difference produced between two electrode tips separated longitudinally by 115 μm , as 5 μA constant current pulses pass axially through the eye. There are three peaks in its profile, within the deepest of which (III) extracellular current flow is reversed, indicated here by the convention of inverting the plot. The approximate actual resistance profile that gives rise to the apparent measured curve in a model of the eye is shown below (R , arbitrary scale). The ERG reaches a large positive maximum near the lamina synapse (LAM) and reverses polarity close to the basement membrane (BM), but the unidirectional gradient of its depth profile down to about 1 mm depth indicates that net extracellular current flow induced by light is directed towards the periphery along most of the length of the receptors. The lower inset gives for comparison the average position of some retinal landmarks determined histologically, although the basement membrane and lamina are probably located about 100 μm more distally in this particular eye.

same time form a perhaps equally specific set of laterally-interacting connections with a quite different set of cells, is avoided with this particular mechanism.

Transretinal resistance was estimated in the outermost 2 mm of the eyes of intact locusts, *Schistocerca gregaria*, by methods similar to those for vertebrate eyes⁷⁻⁹. Isolated, bidirectional pulses of constant current, passed across the retina from a carefully positioned silver harness, produced reasonably uniform lateral fields in the retina. Assuming that current density remains constant with depth, the voltage drop in the axial direction should be proportional to the local radial extracellular resistance. The voltage drops across successive segments of extracellular space were therefore recorded between two glass micropipettes, the tips of which were separated by a fixed depth, and broken to about 20 μm to ensure extracellular recording. Each pipette also recorded the local ERG to a wide field flash. Results were taken as the electrode pair was withdrawn in steps from the eye, as the blunt tips easily snag and distort the tissue on entry.

Figure 1 is an example of the depth profiles consistently obtained, and shows three regions, I, II and III, where the local extracellular voltage drop is large. Unexpectedly, the direction of extracellular current flow in region III is reversed from that found elsewhere, and at the boundaries of III there is no net current flow at all: clearly extracellular current density is not at all uniform with depth, so that the voltage profile does not directly represent the resistance profile. To see what sort of

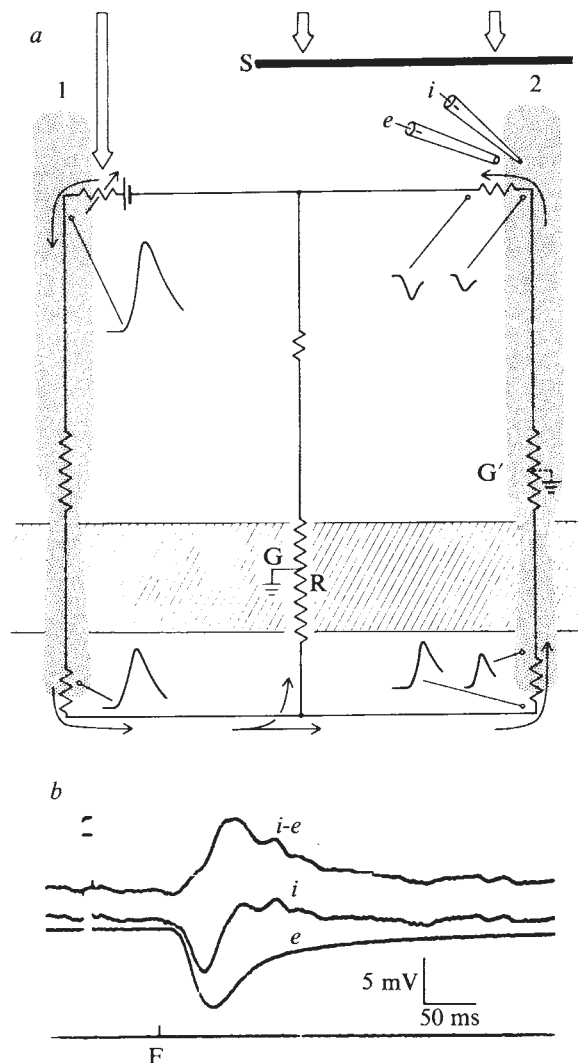


Fig. 2a, Extracellular inhibition of one photoreceptor by another. Photocurrents (small arrows) from excited receptor (1) complete their circuits through nearby unstimulated cells (2), because the orthodox extracellular return path is blocked by a barrier R . The ground reference electrode at G and the virtual ground it induces at G' somewhere in cell 2, cause the recorded waveforms to reverse polarity as shown diagrammatically and measured in Fig. 1. The net local transmembrane voltage difference in cell 2, however, should be hyperpolarising in the axon terminals, suppressing transmitter release, but depolarising in the cell soma. **b**, This last prediction is confirmed in the cell soma of a locust photoreceptor giving a 50 mV response to direct light, but here isolated by a 100 μm diameter opaque mask (S) from a flash (F) which falls on its neighbours. The intracellular record (i) shows an initial negative response followed by a small positive response to scattered light. The simultaneous extracellular response (e) 10 μm away is even more negative, so the net transmembrane effect must be depolarising as predicted above. Electrical subtraction ($i-e$) confirms this.

resistance profile may reproduce this and related anomalies of the response to light and current (S.R.S., unpublished) a model of the retina has been analysed which predicts the data well with a few simple assumptions (described in detail elsewhere). Barrier II is real, and effectively seals off the extracellular space at a point part way along the receptors, starting near the basement membrane. In avoiding this obstruction, most of the applied measuring current crosses into the receptors distally, travels within them, then crosses out again in the lamina. The reversed flow noted in region III consists of this current flowing centrifugally from the lamina to haemolymph channels below the basement membrane, which form a relatively low resistance shunt around the optic lobe. Barrier I represents the progressive loss of measuring current into the

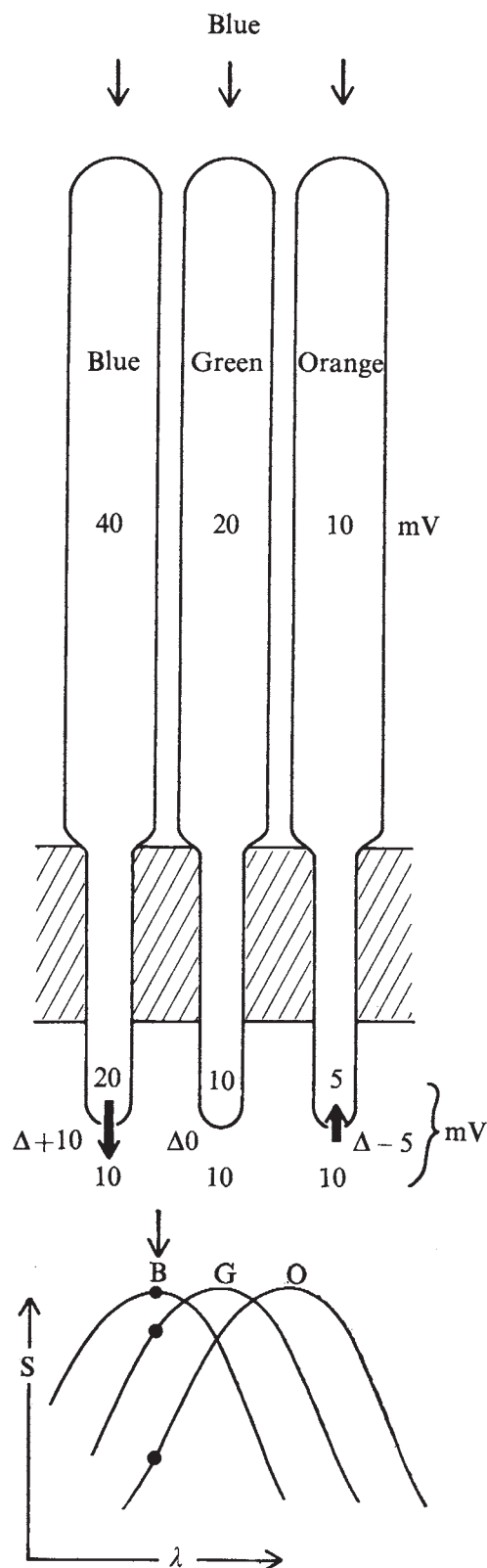


Fig. 3 Selective inter-receptor inhibition through the extracellular space, illustrated arbitrarily with three sets of receptors with differing chromatic responses exposed to blue light (S, sensitivity; λ , wavelength). Blue receptors (B) are excited strongly, thus producing a positive field around the receptor terminals. This field meets potentiometric opposition from the weaker intrinsic responses to blue light of the other two types. With the numbers chosen for illustration, this direct response is just sufficient to counter the inhibitory drive for the green (G) sensitive cells, but transmitter release will be completely suppressed from the terminals of the orange-sensitive set (O), by net inward current. Whatever the particular neuronal sets, suppression will be stronger the greater the difference in response between inhibitor and inhibited.

receptors and is not a barrier at all, and there is probably some increase in resistance with depth, reflecting the decreasing cross section of the eye down to the basement membrane. Barrier III represents the reappearance of this current, and the large voltage drop and depth profile in III indicate that barrier II must extend through region III as well, to span approximately the distance between the basement membrane and the lamina (Fig. 1, R).

The extracellular barrier affects the retinula cells by blocking off the external return path for receptor current which spreads electronically down the axons¹⁰⁻¹². Just as extrinsic currents cross into the receptors to avoid the barrier, so must the returning photocurrents do the same to complete their circuit, since the barriers rectify little. The passive axons of neighbouring unilluminated receptors therefore carry most of the return current from an excited retinula cell (Fig. 2a). This current is directed inwards into these carrier axons, hyperpolarising them, but is directed outwards from their cell bodies back into the extracellular space of the retina, depolarising the somata. Earlier measurements suggested that this last effect may indeed occur¹³, and differential recording has confirmed that the local transmembrane voltage change recorded across a carrier cell soma is depolarising as predicted, and is quite large (Fig. 2b). The early soma depolarisation of about 5 mV gives an estimate of the hyperpolarisation of the terminals which is necessarily a minimum, since current entry is concentrated on the terminal but its exit is distributed over the soma. The system is at least sufficiently powerful for a strongly stimulated group of receptors collectively to suppress transmitter release from their more weakly activated neighbours, to the extent of counteracting voltage changes of several millivolts in their terminals arising from perhaps 10 mV direct depolarisation of their somata.

This presynaptic, electrical inhibitory effect has several interesting consequences. First, because photocurrent will normally spread out tangentially from a single activated receptor terminal in the lamina, the extracellular field created will fall away sharply in the tangential direction from the source. The inhibitory effect of one receptor will in this case be effectively confined to a local circle of near neighbours.

Second, suppose that the eye is illuminated evenly with light, the parameters of which (wavelength for instance) are such as to stimulate one set of cells, B, more than other intermixed sets (Fig. 3). In this case tangential spread of current is minimised because of the uniform tangential voltage field⁷, so that current flow becomes concentrated to produce larger radial fields. Members of set B will produce large intracellular voltages and somewhat smaller extracellular voltages in the lamina (Fig. 3). The latter act as the drive to send currents back up neighbouring axons, but if these are also type B, the drive always sees the larger opposing voltage from the strongly excited Bs: net current flux is thus always outwards (depolarising) in the terminals of the more strongly active set. For other sets, on the other hand, the net current flux could be either outwards or inwards, depending on whether the intrinsic voltage change in the particular terminals exceeds or falls below that of the local lamina drive. In general, for many classes of receptor, the dominant set(s) will suppress most severely those others with the weakest response, and have an intermediate effect on sets in between (Fig. 3). Intrinsic response differences between sets are thus accentuated by this system, but the selectivity of suppression arises not in the wiring, which is the common extracellular space, but simply in the response specificities themselves of the different neural sets, which result in differing potentiometric imbalances at the presynaptic membrane. The singular advantage of this type of selectivity that is difficult to programme with a connectivity-dependent scheme, is that it still functions properly and proportionally if differing attributes such as wavelength and polarisation-plane sensitivity are mixed up in the same neurones¹⁴. A possible drawback is that particular sets of cells cannot be exempted from inhibitory effects, as they could with a selective wiring scheme.

Note that when a particular stimulus activates all the receptors in one area quite strongly, the radial field which develops around the terminals will oppose transmitter release generally in that group. An automatic gain control, able to light adapt and extend the working range of the retina, is built into the first synapse^{15,16}.

Whether the extracellular inhibitory system described above is used to its full potentialities in the arthropod retina is not yet known. It could, for example, refine the rather imperfect polarised light response shown by crustacean photoreceptor somata¹⁷ into a transmitter output showing an almost infinite apparent dichroic ratio, the exact result depending on the presently unknown relationship between transmembrane voltage and transmitter release at these receptor synapses¹¹. In the locust the extracellular inhibition seems to be responsible for the narrowed receptive fields, implying higher acuity, which the lamina cells show over the receptors (ref. 13 and my unpublished work), also observed in the fly and dragonfly^{18,19}. It would be an attractive scheme for use in complex systems of lateral interaction with many cell types, requiring as it does only a regular arrangement of neurones bisected by an extracellular barrier, to develop its specificity. The lack of anatomical connections and the disguised inverted sign of the inhibition in the soma (Fig. 2b) make it difficult to recognise, although it is of course well known at the axon cap of the Mauthner cell^{20,21}.

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- ¹ Hartline, H. K., *Am. J. Physiol.*, **83**, 466–483 (1928).
- ² Burtt, E. T., and Catton, W. T., *J. Insect Physiol.*, **10**, 865–886 (1964).
- ³ Heisenberg, M., *J. exp. Biol.*, **55**, 85–100 (1971).
- ⁴ Braitenberg, V., *Expl Brain Res.*, **3**, 271–298 (1967).
- ⁵ Horridge, G. A., and Meinertzhagen, I. A., *Proc. R. Soc.*, **B175**, 69–82 (1970).
- ⁶ Macagno, E. R., Lopresti, V., and Levinthal, C., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 57–61 (1973); *ibid.*, **71**, 1098–1102 (1974).
- ⁷ Hagins, W. A., Penn, R. D., and Yoshikami, S., *Biophys. J.*, **10**, 380–412 (1970).
- ⁸ Penn, R. D., and Hagins, W. A., *Nature*, **223**, 201 (1969).
- ⁹ Ogden, T. E., and Ito, H., *J. Neurophysiol.*, **34**, 367–373 (1971).
- ¹⁰ Ioannides, A. C., and Walcott, B., *Z. vergl. Physiol.*, **71**, 315–325 (1971).
- ¹¹ Shaw, S. R., *J. Physiol., Lond.*, **220**, 145–175 (1972).
- ¹² Zettler, F., and Järvilehto, M., *J. comp. Physiol.*, **85**, 89–104 (1973).
- ¹³ Shaw, S. R., *Symp. zool. Soc. Lond.*, **23**, 135–163 (1968).
- ¹⁴ Horridge, G. A., *Z. vergl. Physiol.*, **55**, 207–224 (1967).
- ¹⁵ Rushton, W. A. H., *Proc. R. Soc.*, **B162**, 20–46 (1965).
- ¹⁶ Barlow, H. B., and Levick, W. R., *J. Physiol., Lond.*, **200**, 1–24 (1969).
- ¹⁷ Shaw, S. R., *Vision Res.*, **9**, 1031–1040 (1969).
- ¹⁸ Zettler, F., and Järvilehto, M., *Z. vergl. Physiol.*, **76**, 233–244 (1972).
- ¹⁹ Laughlin, S. B., *J. comp. Physiol.*, **92**, 357–375 (1974).
- ²⁰ Furukawa, T., and Furshpan, E. J., *J. Neurophysiol.*, **26**, 140–175 (1963).
- ²¹ Faber, D. S., and Korn, H., *Science*, **179**, 577–8 (1973).

Influenza virus as a teratogen in rhesus monkeys

INFLUENZA virus has been suspected but never proven to be a cause of malformation of the central nervous system in man^{1–4}. One major limiting factor in the evaluation of the teratogenic potential of this and other viral agents has been the absence of a primate model. So far the only studies on the teratogenic effects of this virus have been in the chick embryo and rodents, animals phylogenically distant from *Homo sapiens*^{5–7}. We now report a primate model, the rhesus monkey, showing the teratogenic effect of influenza virus.

We inoculated the virus directly into the foetus, rather than into the mother, to ensure maximum opportunity for the virus to infect the foetus and cause damage⁸. Fourteen pregnant rhesus monkeys (*Macaca mulatta*), serologically negative for influenza A virus antibody, were subjected to laparotomy at 105–111 d gestation, this age having been suggested by the results of a pilot study. At this time influenza A virus was inoculated into the brain of the foetus by passing the inoculating needle through the intact uterine wall and the foetal anterior fontanelle. The inoculum was 0.25 ml (except for one

animal, originally from the pilot study, which has received 0.5 ml) of an Aichi/2/68 WP strain of Hong Kong influenza A virus passaged fifteen times in egg allantoic sac and three times in rhesus kidney tissue culture. This virus titred 10^{8.7} egg infective dose 50% (egg ID₅₀). A further eleven pregnant monkeys without influenza A antibodies were inoculated similarly at 105–108 d gestation with control material: nine received 0.25 ml of the same virus pretreated with specific guinea pig antiserum against K/68AS/494 type strain in influenza A virus (neutralised virus), and two received 0.25 ml of control uninfected allantoic fluid.

Two foetuses inoculated with virus were delivered by caesarean section, respectively, 3 and 11 d after inoculation, and killed immediately. Virus was recovered from the brain, lung, liver, throat and amniotic fluid of both animals, showing that influenza virus replicated in rhesus monkey foetal tissues in our experimental conditions. Having established that infection occurred in the foetuses, we investigated its possible teratogenic effect.

The other 23 foetuses were allowed to reach term (160 ± 5 d). Seven foetuses were delivered spontaneously (four infected and three controls): three of these were stillborn (two infected and one control). The other sixteen foetuses were delivered by caesarean section and all were liveborn (eight infected and eight controls). These animals were killed and examined: fourteen at 1 d (seven infected and seven controls) and one control at 3 d, whereas eight (five infected and three controls) were allowed to live 1 month to determine if any malformation would become more severe with time (this did not seem to be the case).

The virus was isolated in primary rhesus monkey kidney tissue cultures. Haemagglutination-inhibition antibody (HI) titres were determined by standard methods⁹. No virus was isolated from throat swabs taken at delivery from the mothers of either infected or control monkeys, suggesting the absence of persistent maternal infection. In six mothers whose foetuses had been inoculated *in utero* with virus, however, an antibody titre developed and persisted for the period of observation—9–15 months (serum was not available from the other six mothers)—indicating maternal infection. Virus was not recovered from the lung, thymus, spleen, kidney, liver, amniotic fluid or placenta of any of the foetuses. An attempt to isolate virus from the brain of three and the cerebrospinal fluid of eight infected foetuses was also negative. HI antibody titres against influenza A virus were, however, found in the serum of ten of the virus-inoculated foetuses (Table 1). We interpret this as evidence of active antibody production by the foetus, although passive transfer of antibody from the mother across the placenta cannot be excluded. Mothers and foetuses in the control group whose serum was available for testing, with one exception, were negative for influenza A antibody. The exception was an animal which had received incompletely neutralised virus (Table 1).

At necropsy the only macroscopically recognisable malformation was hydrocephalus, present in six of the 12 virus-inoculated animals (Table 1). It was manifest primarily as a

Table 1 Frequency of hydrocephalus and HI titres in rhesus monkey foetuses inoculated with influenza virus and in controls

Animal groups	No. hydrocephalic No. observed	HI titre serum*
Infected group: 12 animals		
Virus inoculated	6/12	1:8–1:512
Control group: 11 animals		
Neutralised virus	0/8	< 1:4
Partly neutralised virus	1/1	1:8
Allantoic fluid	0/2	—

*Serum was not available from two infected animals, one animal in the neutralised virus control group, and the two animals in the allantoic fluid control group.

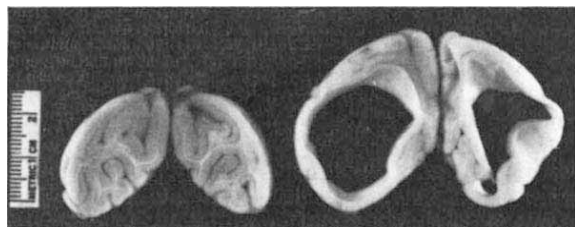


Fig. 1 Corresponding coronal sections of brains of 1-month-old rhesus monkeys after *in utero* intracerebral inoculation of influenza A virus at 107–108 d gestation. Brain on left, inoculated with neutralised virus, appears normal; brain on right, inoculated with virus alone, shows severe hydrocephalus bilaterally.

gross dilatation of the lateral ventricles that was bilateral in all but one case (Fig. 1). The dilatation was consistently most pronounced in the posterior horns of the lateral ventricles, and in one case was limited to a single posterior horn. Hydrocephalus did not occur in the two control animals inoculated with allantoic fluid. Of the nine controls inoculated with neutralised virus, one had hydrocephalus similar to that in the experimental group—unilateral, focal and moderate. This was the only animal inoculated with neutralised virus which developed an antibody titre (Table 1) and its mother had an antibody titre at delivery. This indicates incomplete neutralisation which is well known with influenza virus¹⁰. We believe that the hydrocephalus in this foetus was a sequela of infection with the viable virus in the partially neutralised inoculum.

Severe stenosis or total occlusion of the aqueduct of Sylvius, located between the third and fourth ventricles of the brain, has been reported to be the mechanism of neonatal hydrocephalus induced in the suckling hamster by myxovirus⁷. For this reason we examined the ventricular system of the brain in great detail in five of the six hydrocephalic animals in the infected group. Four had obstructive lesions in either the fourth or third ventricles, or the foramina of Monro, or in combinations. Obstruction of the aqueduct of Sylvius was not observed. On the basis of preliminary histological studies, the obstructive lesions could be classified as sequelae of a destructive ependymitis and/or choroiditis. The precise role of the obstructive lesions in the pathogenesis of the hydrocephalus, however, awaits further investigation.

Thus influenza virus can induce congenital hydrocephalus in the foetus of the rhesus monkey. There may be a similar teratological effect in the human in view of the close phylogenetic relationship to subhuman primates. The demonstration that influenza virus can cross the human placenta and infect the foetus emphasises the need for caution in the use of live vaccines in the pregnant human female¹¹.

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- Coffey, V. P., and Jessop, W. J. E., *Lancet*, ii, 935–938 (1959).
- Saxen, L., Hjelt, L., Siostedt, J. E., Hakosalo, J., and Hakosalo, H., *Acta Path. microbiol. scand.*, 49, 114–126 (1960).
- Coffey, V. P., and Jessop, W. J. E., *Lancet*, i, 748–751 (1963).
- Hakosalo, J., and Saxen, L., *Lancet*, ii, 1346–1347 (1971).
- Hamburger, V., and Habel, K., *Proc. Soc. exp. Biol. Med.*, 66, 608–617 (1947).
- Heath, H. D., Shear, H., Imagawa, D. T., Jones, M. H., and Adams, J. M., *Proc. Soc. exp. Biol. Med.*, 92, 675–682 (1956).

- Johnson, R. T., and Johnson, K. P., *Exptl molec. Path.*, 10, 68–80 (1969).
- Sever, J. L., and London, W. T., *Teratology*, 2, 39–46 (1969).
- Lenette, E. H., and Schmidt, N. J., *Diagnostic procedures for viral and rickettsial infections*, fourth ed., 414–433 (Public Health Association, Inc., New York, 1969).
- Wallis, C., and Melnick, J. L., *J. Virol.*, 1, 478–488 (1967).
- Yawn, D. H., Pycatte, J. C., Joseph, J. M., Eichler, S. L., and Garcia-Bunuel, R., *J. Am. med. Ass.*, 216, 1022–1023 (1971).

Non-encephalitogenic synthetic analogues of the determinant for allergic encephalomyelitis in guinea pigs

EXPERIMENTAL allergic encephalomyelitis (EAE) is an auto-immune demyelinating disease induced by sensitisation to whole central nervous system (CNS) tissue homogenates, to myelin basic protein (BP) or to peptide regions derived from BP^{1–4}. One such region, peptide E, was isolated from the pepsin digest of the BP and induces EAE in guinea pigs^{3,5}. The shortest segment of peptide E which induces EAE, H-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-OH (peptide S3), has been synthesised by the Merrifield procedure⁶. Studies with analogous synthetic peptides with single amino acid residue substitutions have shown that the linearly spaced array of tryptophan, glutamine and lysine is needed to induce EAE⁶. We have now determined the minimum substitution in the amino acid sequence of peptide S3 necessary to destroy encephalitogenicity and to preserve the sequence for delayed hypersensitivity (DTH), also reported for peptide S3⁷. The sequence requirement for induction of EAE is not restricted to tryptophan, glutamine and lysine, and certain analogous peptides retain the ability to induce and elicit DTH unaccompanied by signs of EAE.

All peptides were synthesised from Boc-glycine-resin ester containing 0.25–0.50 μmol amino acid per g resin (Schwarz/Mann) using an adaptation of the Merrifield solid phase method⁸. Details of the synthesis of peptide S3 and its analogues, including methods of coupling, cleavage and purification have been described before⁹. High voltage electrophoresis or paper chromatography of each purified peptide showed a single spot with ninhydrin stain. Amino acid analyses of samples hydrolysed at 110 °C for 24 h with 6N HCl gave values close to whole integers of amino acids expected for each particular sequence.

All EAE assays were carried out in Rockefeller-Hartley guinea pigs (500–600 g). Each peptide was tested in doses of 33.3–1,000 μg , as was the encephalitogenic BP from bovine myelin. Each antigen was administered in a single injection of 0.1 ml, made up in saline emulsified with an equal volume of Freund's complete adjuvant (FCA) (Difco). Animals that developed severe clinical signs of EAE were killed immediately and all others were killed after 30 d. Brain and spinal cord tissues were examined histologically for lesions characteristic of EAE².

The DTH assay was performed on day 11 after sensitisation. The skin test antigen was administered intracutaneously in 0.1 ml of sterile saline as described previously⁷, and the injection site was inspected after 1, 3, 4 and 24 h. The cross sectional diameter of the erythematous area was recorded after 24 h.

The amino acid sequences of the peptides used are shown in Table 1 and the EAE and DTH assays are summarised in Table 2. The presence of lesions in animals receiving 33.3 μg and 100 μg corresponded with the clinical manifestation of the disease.

The DTH skin response to 0.10 μmol of each peptide antigen ranged between 9×10 and 12×13 mm. Positive skin tests were confirmed histologically. Thin sections of the injected site together with adjacent areas showed extensive plasma cell infiltrates indistinguishable from the delayed skin response to BP. Neither the delayed skin response nor the size of the erythematous area differed significantly when guinea pigs were sensitised and skin tested with the BP or any antigen listed in Table 1. Ovalbumin and bovine serum albumin, used for control skin test antigens, gave negative results.

Our results confirm that phenylalanine or valine substituted

Table 1 Amino acid sequence of synthetic peptides

Laboratory code no.												No. of substituted residues
		1	2	3	4	5	6	7	8	9	10	
1	S3 (peptide E)	H-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Gly-OH										None
2	S31	H-Phe-Ser-Phe-Gly-Ala-Glu-Gly-Gln-Lys-Gly-OH										3
3	S37	H-Phe-Ser-Val-Gly-Ala-Glu-Gly-Gln-Lys-Gly-OH										3
4	S38	H-Gly-Gly-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Gly-OH										1, 3
5	S35	H-Ala-Ser-Trp-Gly-Gly-Gly-Gly-Gln-Lys-Gly-OH										1, 5, 6
6	S23	H-Phe-Ser-Trp-Gly-Ser-Leu-Pro-Gln-Lys-Gly-OH										5, 6, 7
7	S25	H-Phe-Ser-Tyr-Gly-Ala-Glu-Gly-Gly-Lys-Gly-OH										3

The amino acid sequence of synthetic peptides used in the study are shown. From 150–225 mg of each peptide antigen were prepared. The recovery ranged from 40–60% of theoretical yield.

for tryptophan destroys encephalitogenicity⁶, and have shown that tyrosine substituted for tryptophan renders the peptide EAE-inactive. This underlines the importance of the tryptophyl residue in preserving the encephalitogenic function of the peptide. But this role of tryptophan can be emphasised only in relationship to other amino acids of the sequence of peptide S3. The presence of tryptophan in its required position in the sequence, accompanied by glycine-glycine substituted for phenylalanine-serine (peptide S38) or glycine-glycine substituted for alanine-glutamic acid (peptide S35) did not retain encephalitogenicity. Peptide S23 contained tryptophan, glutamine, and lysine at their native positions, but other substitutions destroyed its encephalitogenicity.

It is interesting that tyrosine is an essential component of a structurally similar determinant, H-Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-OH, (residues 65–74 of the BP molecule¹⁰) which induces EAE in rabbits⁴. Tyrosine is at a position identical to that of tryptophan, separated by four residues from the C-terminal sequence of glutamine-lysine. The nature of these four residues is important because peptide S23, with serine-leucine-proline substitutions at positions five, six and seven and intact N- and C-terminal sequences, did not retain the activity of peptide S3. Thus each component amino acid has a role in the activity of peptide S3. The requirement for tryptophan in the encephalitogenic sequence cannot be attributed to the aromatic nature of its side chain, since phenylalanine or tyrosine substitutions, as in peptides S31 and S25 respectively, did not fulfil the requirement for encephalitogenicity. Thus the determinant has a unique chemical structure, and any amino acid substitution alters its biological function.

Although amino acid substitutions destroyed the encephalito-

genic of the determinant, the DTH response was not abolished. The peptides retained the capacity to induce and elicit DTH responses in guinea pigs sensitised with encephalitogenic or non-encephalitogenic antigens. The preparation of non-encephalitogenic analogues of the EAE-inducing determinant bearing structural specificity recognised by animals sensitised with BP or peptide S3 provides antigens for the study of prevention, suppression and treatment of EAE.

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- 1 Paterson, P. Y., *Adv. Immun.*, **5**, 131–208 (1966).
- 2 Eylar, E. H., Saik, J., Beveridge, G. C., and Brown, L. V., *Archs Biochem. Biophys.*, **132**, 34–48 (1969).
- 3 Hashim, G. A., and Eylar, E. H., *Archs Biochem. Biophys.*, **129**, 645–654 (1969).
- 4 Shapira, R., Chou, F. C. H., McKneally, S., Urban, E., and Kibler, R. F., *Science*, **173**, 736–738 (1971).
- 5 Eylar, E. H., and Hashim, G. A., *Proc. natn. Acad. Sci. U.S.A.*, **61**, 644–650, (1968).
- 6 Westall, F. C., Robinson, A. R., Caccam, J., Jackson, J., and Eylar, E. H., *Nature*, **229**, 22–24 (1971).
- 7 Hashim, G. A., Hwang, F., and Schilling, F. J., *Archs Biochem. Biophys.*, **156**, 298–309 (1973).
- 8 Gutte, B., and Merrifield, R. B., *J. biol. Chem.*, **246**, 1922–1941 (1971).
- 9 Hashim, G. A., and Sharpe, R., *Immunochimistry*, **11**, 633–640 (1974).
- 10 Eylar, E. H., Brostoff, S., Hashim, G. A., Caccam, J., and Burnett, P., *J. biol. Chem.*, **246**, 5770–5784 (1971).

Table 2 Biological properties of synthetic analogues of the determinant for allergic encephalomyelitis in guinea pigs

Sensitising antigen dose	33.3	100 (µg in FCA)	333	1,000	DTH
S3 (peptide E)	10/13	5/5	2/8	3/8	+
S31	0/8	0/16	0/4	0/4	+
S37	0/5	—	0/5	0/5	+
S38	0/5	0/5	0/10	0/10	+
S35	0/4	—	0/4	0/4	+
S23	0/5	—	0/5	0/5	+
S25	0/5	0/4	0/5	0/5	+
Bovine basic protein	15/18	16/21	4/4	4/4	+

The sensitising antigens were injected in 0.1 ml FCA emulsion at the above concentrations in the hind foot pad. Sensitised animals were skin-tested for delayed type hypersensitivity (DTH) at day 11 after sensitisation. The lowest dose of peptide S3 and bovine basic protein was 25 µg rather than 33.3 µg.

Inhibition of leukocyte locomotion and chemotaxis by lipid-specific bacterial toxins

SINCE a number of cytolytic bacterial protein toxins have specific actions on individual membrane lipids, these toxins, used at sub-toxic doses, should be useful for exploring the role of lipids in membrane-activated cell functions. I have suggested^{1–3} that leukocyte chemotactic factors initiate a locomotor response by virtue of their ability to penetrate the hydrophobic interior of the lipid bilayer of the cell membrane, and thus possibly to bring about changes in the intracytoplasmic concentration of divalent cations and activation of contractile systems in the cytoplasm. If this is so, it may be possible to modify locomotor and chemotactic responses in leukocytes using lipid-specific toxins. The oxygen-labile lysins such as streptolysin O or *Clostridium perfringens* θ toxin show a specific affinity for cell membrane cholesterol. Bacterial phospholipases C attack the polar heads of a number of membrane phospho-

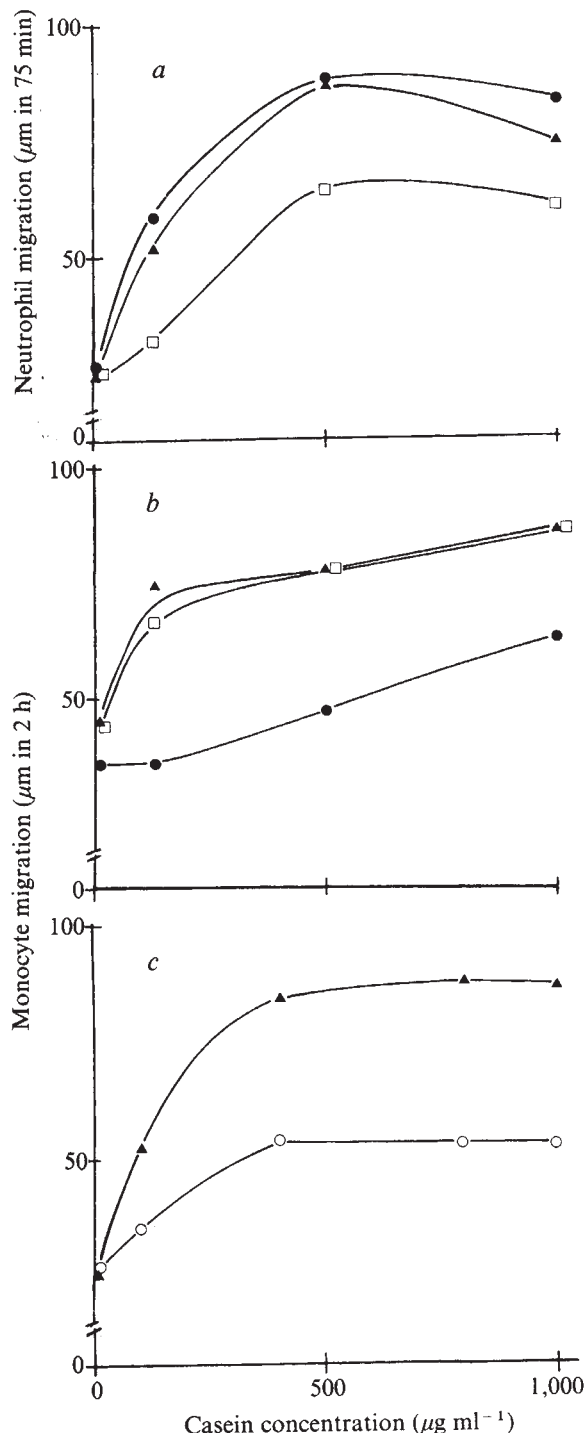


Fig. 1 *a*, Dose-response curves of human neutrophils to casein in the presence of *Cl. perfringens* PLC (200 mU ml^{-1}) (●), or θ toxin (0.3 HU ml^{-1}) (□), or in the absence of added toxin (▲). PLC activity was determined by Dr R. Möllby as described previously⁸. θ Toxin was reduced with 2-mercaptoethanol and assayed by haemolytic titration against 1% sheep cells. Toxins were added to the chemotactic chambers at equal concentrations on both sides of the filter immediately before incubation. In other experiments leukocytes were reincubated with toxin then washed. Inhibition similar to that shown in the figure was observed. *b*, Dose-response curves of human monocytes to casein in the presence of PLC (200 mU ml^{-1}) (●), θ toxin (0.3 HU ml^{-1}) (□), or untreated (▲). Conditions of assay as in *a*. Monocytes preincubated with PLC then washed free of toxin also showed inhibition of locomotion to casein. *c*, Dose-response curves of human monocytes in the presence (○), and absence (▲) of sphingomyelinase C (400 HU ml^{-1}). Conditions of assay as in *a*. The toxin was assayed by hot-cold haemolysis¹⁵ by Dr Christine McCartney. Fig. 1*a-c* shows leukocyte migration in a positive gradient, that is, from zero concentration of casein above the filter to the stated concentration below it.

lipids⁴. Among those phospholipases, sphingomyelinase C, the β toxin of *Staphylococcus aureus*, shows a high substrate specificity for sphingomyelin⁵. Here I show that the locomotor responses of leukocytes to chemoattractants are modified by these toxins and that neutrophils show a different pattern of response from monocytes. The locomotor response of human blood monocytes is inhibited by *Cl. perfringens* phospholipase C (PLC) and by sphingomyelinase C but not by *Cl. perfringens* θ toxin. In contrast, the response of human blood neutrophils is inhibited by θ toxin but not by phospholipases C.

Human blood neutrophils and monocytes were obtained from normal venous blood and separated from one another by centrifugation on Ficoll-trisil after preliminary sedimentation of the red cells with dextran. Leukocyte locomotion and chemotaxis were measured by the micropore filter method as described previously⁶ by determining the distance migrated by the leading front of cells⁷. The distinction between oriented and non-oriented migration was made by varying the concentrations of chemotactic factor above and below the filter and calculating the deviation from expected random migration as described by Zigmond and Hirsch⁷. The toxins were pure⁸ and not contaminated with one another; an important point since commercial samples of PLC which have been reported to influence leukocyte function, may contain θ toxin⁹, and commercial PLC had θ -like effects in the experiments reported here. Sphingomyelinase C was prepared from *Staphylococcus aureus*¹⁰ and contained a trace of nuclease which does not affect chemotaxis (P. C. W., unpublished). The viability of leukocytes after toxin treatment was checked by direct observation of morphological changes and by Trypan blue exclusion. No cytotoxic effects were seen at doses of PLC below 400 mU ml^{-1} , of θ toxin below 1 haemolytic unit (HU) ml^{-1} or of sphingomyelinase C at any dose tested ($< 10^4 \text{ HU ml}^{-1}$).

The toxins had little effect on unstimulated random leukocyte locomotion but they inhibited the ability of leukocytes to respond to the presence of a chemotactic factor by increasing the speed of their locomotion. This inhibition was seen not only when cells were migrating in a chemotactic gradient but also when the concentration of the factor was uniform throughout the system, which suggests that the toxins act on the mechanism of locomotion itself rather than on the mechanism by which a cell becomes orientated in a gradient. The effect of PLC and of θ toxin on the migration of human neutrophils towards casein at varying doses is shown in Fig. 1*a*. θ Toxin inhibited the migration of neutrophils towards casein and other chemotactic factors such as endotoxin-activated serum and alkali-denatured human serum albumin (HSA). In contrast, PLC had no inhibitory effect on neutrophil locomotion (Fig. 1*a*). Addition of free cholesterol to the medium prevented the inhibitory action of θ toxin on neutrophil locomotion (Table 1).

The effect of the same toxins on migration of human blood monocytes is shown in Fig. 1*b*. PLC inhibited the migration of monocytes towards casein but θ toxin at sub-toxic doses had no effect. These results were thus the opposite of those obtained with neutrophils. PLC slightly inhibited the migration of unstimulated monocytes but inhibited the migration of chemically stimulated cells more strongly. The effects of sphingomyelinase C resembled those of PLC (Fig. 1*c*). It inhibited the response of monocytes to casein or to alkali-denatured HSA at doses of enzyme down to 1 HU ml^{-1} but did not inhibit monocyte migration in the absence of a chemotactic factor. Sphingomyelinase C was without effect on neutrophil locomotion.

The fact that lipid-specific bacterial toxins inhibit the chemically directed locomotion of leukocytes supports the hypothesis that interactions of chemotactic factors with membrane lipids are of importance in initiating this locomotion. The differences in responsiveness between neutrophils and monocytes raise some interesting questions. θ toxin, like streptolysin O¹¹, another cholesterol binding toxin, inhibits the response of neutrophils but not monocytes to chemotactic factors, which suggests that cell membrane cholesterol may function in the

Table 1 Effect of cholesterol on the inhibition of human blood neutrophil migration by θ toxin

	Neutrophil migration (μm in 75 min)	
	No casein	Casein 800 $\mu\text{g ml}^{-1}$ below filter
Untreated neutrophils	25	99
Neutrophils treated with θ toxin 0.3 HU ml^{-1}	24	52
Neutrophils treated with θ toxin 0.3 HU ml^{-1} and cholesterol 10^{-4} M		95

interaction of such factors with neutrophils but less so with monocytes. The inhibition of monocyte migration obtained with sphingomyelinase C suggests that in the monocyte the interaction involves sphingomyelin. It is probably relevant that, from published data for human¹² and other¹³ cells, the molar ratios of cholesterol to sphingomyelin and of cholesterol to phosphatidylcholine are about twice as high in neutrophil membranes as in those of monocytes or macrophages, but it will not be possible to be more precise about these interactions until more is known about the lipid composition of human leukocyte plasma membranes and how these lipids interact with proteins. There may be species differences too, since the inhibitory effect of streptolysin O on neutrophil chemotaxis varies from species to species¹⁴. The actions of membrane-active bacterial toxins of good purity on other leukocyte functions require further study. These proteins should prove useful not only as probes of the role of individual lipids in membrane function; their effects are also pertinent to the infective process in bacterial disease.

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- Wilkinson, P. C., *Nature*, **244**, 512–513 (1973).
- Wilkinson, P. C., *Nature*, **251**, 58–60 (1974).
- Wilkinson, P. C., in *Primitive Sensory and Communication Systems: the Taxes and Tropisms of Microorganisms and Cells* (edit. by Carille, M. J.) (Academic, New York, in the press).
- Bernheimer, A. W., in *Microbial Toxins* (edit. by Ajl, S. J., and Montie, T. C.), 184–212 (Academic, New York, 1970).
- Doery, H. M., Magnusson, B. J., Gulaskharan, J., and Pearson, J. E., *J. gen. Microbiol.*, **40**, 283–296 (1965).
- Wilkinson, P. C., *Chemotaxis and Inflammation* (Churchill-Livingstone, Edinburgh, 1974).
- Zigmond, S. H., and Hirsch, J. G., *J. exp. Med.*, **137**, 387–410 (1973).
- Möllby, R., and Wadström, T., *Biochim. biophys. Acta*, **321**, 569–584 (1973).
- Möllby, R., Nord, C.-E., and Wadström, T., *Toxicon*, **11**, 139–147 (1973).
- Wadström, T., and Möllby, R., *Biochim. biophys. Acta*, **242**, 288–307 (1971).
- Andersen, B. R., and van Epps, D. E., *J. infect. Dis.*, **125**, 353–359 (1972).
- Gottfried, E. L., *J. Lipid Res.*, **8**, 321–327 (1967).
- Mason, R. J., Stossel, T. P., and Vaughan, M., *J. clin. Invest.*, **51**, 2399–2407 (1972).
- Van Epps, D. E., and Andersen, B. R., *Infect. Immun.*, **9**, 27–33 (1974).
- Low, D. K. R., Freer, J. H., Arbuthnott, J. P., Möllby, R., and Wadström, T., *Toxicon*, **12**, 279–285 (1974).

synergistic effect of drugs and antibody uncombined, since there is a high probability of *in vivo* dissociation^{4–6}. Although this synergistic drug-antibody effect has been studied in detail^{7–9}, the original aim of preparing a stable drug-antibody conjugate has also been pursued and evidence of *in vivo* tumour suppression has been obtained with covalent conjugates of rabbit antitumour immunoglobulin (Ig) and alkylating agents^{10,11}. In these studies, direct conjugation of the drugs with Ig was carried out using a water-soluble carbodiimide. Successful tumour suppression was, however, dependent on using large amounts of linked material since severe limitations are imposed on the degree of drug substitution by the physico-chemical changes induced in the immunoglobulin by linkage. These changes are reflected in loss of antibody activity and poor water solubility of the preparations thus limiting the clinical potential of complexes in this form. Here we describe a method of overcoming these problems. Instead of direct substitution of the drugs on the Ig, an intermediate carrier molecule is heavily substituted and the drug carrier then linked to Ig. By minimising interference with the chemical structure of Ig in this linkage step, a conjugate is produced with both a high concentration of drug and little loss of antibody activity. This conjugate has proved effective in suppressing tumour growth in mice.

It was planned to attach drug to carrier by means of a carbodiimide induced carboxamide bond and therefore suitable compounds were sought containing free carboxyl or free amino groups. Since many polycations are toxic¹², a polyanion—polyglutamic acid (PGA)—was chosen as the carrier and *p*-phenylenediamine mustard (PDM), with its free amino group as the drug. The PGA was poly-L- α -glutamic acid, molecular weight, 35,000 (Miles Seravac) and the PDM, *N,N*-bis-(2-chloroethyl)-*p*-phenylenediamine, was supplied by the Chemical Defence Establishment, Porton. The drug-carrier complex PDM-PGA was prepared by reacting 250 mg of PGA in aqueous solution at 10 mg ml^{-1} with 1 g EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and 14 ml of PDM (10 mg ml^{-1}) at room temperature. The PDM-PGA was isolated by precipitation and washing at pH 4.0 in the cold, and finally redissolved by addition of 1 N NaOH. Evidence of a covalent conjugate came from a shift in the ultraviolet absorption spectrum of PDM after linkage from $\lambda_{\text{max}}_{258\text{nm}}$ to $\lambda_{\text{max}}_{277\text{nm}}$ and from various tests showing that the PDM and PGA behaved as one entity in conditions of dialysis, gel filtration and ion-exchange chromatography. Determination of alkylating activity of the PDM-PGA was by a modification of the Epstein¹³ test in which the reaction temperature was reduced to 80 °C. From this test and results of ultraviolet spectroscopy the substitution ratio of mol PDM per mol PGA was calculated to be 45:1, representing a substitution of approximately 16% of the available carboxyl groups of PGA.

PDM-PGA was coupled to Ig from a rabbit antiserum against mouse lymphoma cells (EL4) made specific by repeated absorption with normal mouse spleen cells¹⁴. Linkage was effected by a carbodiimide reaction between free carboxyl groups on the drug carrier and amino groups on the Ig using the following 'concentration-dilution' technique. PDM-PGA was concentrated to 200 mg ml^{-1} by membrane ultrafiltration and EDC added to a final concentration of 40 mg ml^{-1} . After 2 min at room temperature this mixture was diluted 40-fold with phosphate-buffered saline (pH 7.2) and added to an equal volume of Ig at 5 mg ml^{-1} . Excess EDC was quenched by addition of sodium acetate to a final concentration of 0.15 M. The mixture was dialysed against cold saline to remove unwanted low molecular weight components and finally re-concentrated by membrane ultrafiltration. Analysis of the complex gave values for PDM-PGA-Ig of 2.8:10 mg ml^{-1} respectively. A variety of physicochemical tests were carried out to show that coupling had taken place. These include ethanol precipitability, ion-exchange chromatography and immunoelectrophoresis. Antibody activity of the complex was determined by a ⁵¹Cr-release microcytotoxicity assay¹⁴. EL4

Suppression of tumour growth in mice by a drug-antibody conjugate using a novel approach to linkage

THE possibility of using cytotoxic drugs linked to tumour-specific antibodies as a form of cancer chemotherapy has been revived recently. In theory the antibodies would selectively transport the drugs to the target tumour site. The demonstration by Ghose *et al.*^{1,2} and also by Flechner³ that chlorambucil and antibody combined produce an augmented anti-tumour effect can, however, be explained by an additive or

Table 1 Survival of C57BL/6 mice bearing EL4 lymphoma

Daily treatment (intraperitoneal) (days 1-4)	Median survival time* (d)	Mice alive free of tumour at day 60
0.4 ml saline	13	0/5
0.4 ml Ig (4 mg)	19	0/5
0.4 ml PDM-PGA (0.75 mg PDM)	25	1/5
0.4 ml Ig (4 mg)+0.4 ml PDM-PGA (0.75 mg PDM)	38	2/5
0.4 ml PDM-PGA-Ig (0.75 mg PDM, 4 mg Ig)	> 100	5/5

*Median survival time as defined in ref. 16 is the time to which 50% of the animals in the group survive.

cells were used as targets and were incubated for 3 h with guinea pig serum as complement source. C57BL/6 lymph node cells (strain of origin of tumour) were used as a control for tumour specificity. From such tests it was shown that 66% of the original anti-EL4 activity was retained in the complex and there was no loss of specificity.

To show the effectiveness of the complex as an antitumour agent *in vivo*, several groups of five C57BL/6 mice were inoculated with 5×10^4 live EL4 cells intraperitoneally. This is approximately 10,000 times the LD₅₀ challenge dose. After 24 h the mice received the first of four daily injections of the complex or control preparations. Table 1 shows the results of such an experiment. A small increase in survival time was obtained with the Ig alone and with the PDM-PGA alone. When PDM-PGA and Ig were given together, uncombined, the mice survived nearly three times as long as the mice treated with saline—a drug-antibody effect. The greatest increase in survival was obtained, however, when the complex was given, no mice having died by day 60.

PDM has also been conjugated to glutamyl-containing synthetic polypeptides by carbodiimides in a study of the use of macromolecules as carriers for cytotoxic groups¹⁵. In those experiments although the toxicity of the PDM was markedly reduced on conjugation, little improvement in therapeutic efficiency was obtained. No attempt was made to direct the drug to the tumour target by linking those preparations to tumour-specific antibody. In our study, PDM conjugated to PGA, produces a material with a similarly reduced toxicity. (LD₅₀ of ~200 compared with LD₅₀ of 5 for free PDM.) This material on linkage to Ig containing tumour-specific antibody produced a complex with powerful antitumour properties. The greater effectiveness of the complex compared with the PDM-PGA plus Ig unlinked, provides evidence that the complex is stable and suggests that a homing mechanism and not a drug-antibody effect is operating.

How such a complex exerts its cytotoxic action is not yet known. It may be sufficient to alkylate cell surface components for cell death to occur. Alternatively, enzymes associated with the cell membrane could release the drug from the conjugate and allow entry of free drug into the cell. Another possibility is that the entire conjugate is pinocytosed to exert its action within the cell.

The concept of linking cytotoxic drugs to antibody through an inert intermediate carrier offers a wide scope for improved cancer chemotherapy in the future, with the possibility of using a variety of drugs, different carriers and antibody preparations of greater purity.

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¹ Ghose, T., *et al.*, *Br. med. J.*, **3**, 495-499 (1972).

² Ghose, T., and Nigam, S. P., *Cancer*, **29**, 1398-1400 (1972).

³ Flechner, I., *Eur. J. Cancer*, **9**, 741-745 (1973).

- ⁴ Ross, W. C. J., *Chem-Biol Interactions*, **8**, 261-267 (1974).
⁵ Davies, D. A. L., and O'Neill, G. J., *Br. J. Cancer*, **28**, Supp. 1, 285-298 (1973).
⁶ Rubens, R. D., and Dulbecco, R., *Nature*, **248**, 81-82 (1974).
⁷ Davies, D. A. L., Buckham, S., and Manstone, A. J., *Br. J. Cancer*, **30**, 305-311 (1974).
⁸ Davies, D. A. L., *Cancer Res.*, **34**, 3040-3043 (1974).
⁹ O'Neill, G. J., Pearson, B. A., and Davies, D. A. L., *Immunology* (in the press).
¹⁰ Davies, D. A. L., and O'Neill, G. J., *Proc. XI Int. Cancer Congress Florence* (1974) (in the press).
¹¹ O'Neill, G. J., and Davies, D. A. L., *Proc. second National Congress of Oncology Bucharest* (1975) (in the press).
¹² Silman, H. I., and Sela, M., in *Poly- α -amino acids* (edit. by Fasman, G. D.), 657 (Edward Arnold, London, 1967).
¹³ Epstein, J., Rosenthal, R. W., and Ess, R. J., *Analyt. Chem.*, **27**, 1435-1439 (1955).
¹⁴ Davies, D. A. L., Manstone, A. J., and Buckham, S., *Br. J. Cancer*, **30**, 297-304 (1974).
¹⁵ Szekerke, M., Wade, R., and Whisson, M. E., *Neoplasia*, **19**, 199-209 (1972).
¹⁶ *Cancer Chemotherapy Reports*, **3**, No. 2 (1972).

Inhibition of murine plasmacytoma TEPC 15 by its specific antigen

In favourable conditions hosts can produce antibodies to components of their tumours. An intriguing example is the ability of BALB/c mice immunised with syngeneic myeloma proteins MOPC 315 or MOPC 460 (ref. 1) to make antibodies to the idiotypic determinants of these proteins. The sensitised mice reject tumour-producing MOPC 315 or MOPC 460 cells; this may result from reaction of anti-idiotypic antibodies with myeloma proteins on myeloma cell surfaces. On the assumption that any substance able to bind to a cell membrane surface component could modify the rate of cell division, we hoped to inhibit myeloma growth by injecting specific antigen into the hosts. This plan was based on the observation that specific antigen binds to the cell surface of antibody-secreting myeloma cells². We describe here the inhibition of the growth of plasmacytoma TEPC 15 by the specific pneumococcus C polysaccharide (PCP) antigen.

Murine plasmacytomas are transplantable in mice and secrete a specific clonal immunoglobulin. TEPC 15 excretes an IgA immunoglobulin which specifically binds PCP³. MOPC 315 excretes another IgA immunoglobulin which binds both dinitrophenyl (DNP) and trinitrophenyl groups⁴. Both tumours were adapted to grow as ascites cells after intraperitoneal injection; they also induced tumours in the peritoneum. Both tumours have membrane-bound immunoglobulin with the same respective antigen specificities.

PCP after purification^{5,6} contained phosphorylcholine and could thus be considered a specific antigen (and phosphocholine the specific hapten) for TEPC 15. Conversely, we used DNP-bovine gamma globulin (BGG-DNP, 38 residues per BGG molecule) as the specific antigen (and 2, 4-dinitrophenol as specific hapten) for MOPC 315. Ascites cells were obtained in 8-12-week-old SJBALB/c mice by one intraperitoneal injection of 5×10^4 cells for TEPC 15 and 3×10^4 cells for MOPC 315. These tumour cells were obtained in ascitic fluid in densities of $2-20 \times 10^6$ ml⁻¹, with a viability of 90-98% as tested by trypan blue exclusion. Fewer cells of MOPC 315 than TEPC 15 were needed to induce ascites and tumours, and the former grew much faster than the latter. In most cases we injected cells and

Table 1 TEPC ascites in mice treated twice a week

Injection	Treatment	Mice with tumours	
		4th week	6th week
5×10^4 cells	Saline	20/25	22/25
	10 μ g PCP	7/20	15/25
	10 μ g Dextran	9/15	12/15
	10 μ g BGG-DNP	9/10	10/10

Mice were injected intraperitoneally with 5×10^4 cells in 0.2 ml and received either saline for controls or antigen treatment in 0.2 ml by the same route. Then twice a week they again received intraperitoneally, the same treatment without cells. The mice were killed and autopsied on week 4 for one experiment, and on week 6 for the other. Mice without tumours or ascites are recorded as 0; mice with either tumour or ascites are recorded as 1.

antigen or hapten intraperitoneally on day 1. Subsequently, we injected the antigen or the hapten twice a week. Mice were killed for necropsy at the times shown in the legend to Table 1.

Table 1 shows that, whereas 20 out of 25 control mice had ascites and tumours by week 4 and 22 of them had lesions by week 6, only 7 out of 20 receiving 10 µg of PCP twice a week developed lesions. Results not shown here indicate that this effect is dose responsive above the minimal concentration of 1 µg. This inhibition of tumour growth could be the result of adjuvant-like activity. Dextran which is also a polysaccharide, and known to be an adjuvant⁷, was tested for anti-tumour activity. Table 1 shows that tumour development in mice treated with dextran was no different from that in controls.

BGG-DNP (the specific antigen for MOPC 315) had no

Table 2 MOPC 315 ascites in mice treated once a week

Injection	Treatment	Mice with tumours (size of lesions)	
		2nd week 11/12 (Large tumours)	4th week 12/12 (Large tumours)
2 × 10 ⁴ cells	Saline	8/15 (Small tumours)	12/12 (6 Small tumours 6 Large tumours)
	10 µg BGG-DNP	14/15 (Large tumours)	15/15 (Large tumours)
	10 µg PCP		

As in Table 1, antigens were injected once a week. Large tumours had mean diameters >6mm. Small tumours had mean diameters <2mm.

effect on the growth of TEPC 15, but it did inhibit growth of MOPC 315 (Table 2). Although 11 out of 12 control mice had large tumours after 2 weeks, only eight out of 15 treated with BGG-DNP had lesions, and 14 out of 15 treated with PCP had lesions. Moreover, the lesions found in the animals given BGG-DNP were much smaller than the others. The situation was similar on week 4. Whereas specific antigens had a specific inhibitory effect, the related haptens had no effect although we used large doses: neither phosphorylcholine nor dinitrophenol slowed the growth of TEPC 15 or MOPC 315 (Table 3).

Table 3 Comparative activities of specific haptens and antigens on ascites growth

Plasmacytomas	Treatment	Mice with tumours	
		Week 2	Week 4
TEPC 15 5 × 10 ⁴ cells	Saline	5/10	9/10
TEPC 15 5 × 10 ⁴ cells	2 µg Phosphorylcholine	4/5	5/5
TEPC 15 5 × 10 ⁴ cells	10 µg PCP	1/10	5/10
MOPC 315 2 × 10 ⁴ cells	Saline	11/12	11/12
MOPC 315 2 × 10 ⁴ cells	10 µg DNP	11/11	9/9
MOPC 315 2 × 10 ⁴ cells	10 µg BGG-DNP	6/14	9/13

As in Table 1, antigens were injected twice a week.

We do not know the mechanism of this inhibitory effect. The host immune system does not seem to be involved because at such high doses, pneumococcal polysaccharides are tolerogenic^{8,9}, even in a single dose⁹. Thus we favour a direct effect of the antigen on tumour cells at the membrane level. Because the IgA immunoglobulins secreted by both plasmacytomas are unable to bind complement, we assume that the inhibitory effect is not mediated by the complement. Concanavalin A has been shown to bind to the B lymphocyte cell membrane, resulting in capping and leading ultimately to blastogenesis and synthesis of IgM^{11,12}. Antigens also induce capping and blastogenesis in immunogenic doses, whereas such phenomena are inhibited by tolerogenic doses¹³. Furthermore, low doses of mitogens can stimulate the membrane Na⁺,K⁺-stimulated ATPase^{14,15} and

5'-nucleotidase activity, although the latter is inhibited by high doses¹⁶. Thus we suggest that the PCP antigen sticks to the tumour cell surface, leading to structural and enzymatic changes in the cell membrane which in turn inhibit cell division.

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- Lynch, R. G., Graff, R. V., Sirisinha, S., Simms, E. S., and Eisen H. N., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1 540-1 544 (1972).
- Hannestad, K., Kao, M. S., and Eisen, H. E., *Proc. natn. Acad. Sci. U.S.A.*, **69**, (2) 295-2 299 (1972).
- Leon, M. A., and Young N. M., *Biochemistry*, **10**, (1) 424-1 429 (1971).
- Eisen, H. N., Simms, E. S., and Potter, M., *Biochemistry*, **7**, (4) 126-4 134 (1968).
- Tomasz, A., *Science*, **157**, 694-697, (1967).
- Brindish, D. E., and Baddiley, J., *Biochem. J.*, **110**, 573-582 (1968).
- Battisto, J. R. and Pappas, F., *J. exp. Med.*, **138**, 176-193 (1973).
- Neeper, C. A., and Seastone, C. V., *J. Immun.*, **93**, 867-871 (1964).
- Siskind, G. W., and Howard, J. G., *J. exp. Med.*, **124**, 417-429 (1966).
- Siskind, G. W., *Ann. N.Y. Acad. Sci.*, **181**, 9-17 (1971).
- Unanue, E. R., Perkins, W. D., and Karnovsky, M. J., *J. exp. Med.*, **136**, 885-906 (1972).
- Andersson, J., and Melchers, F., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 416-420 (1973).
- Diener, E., and Paetkau, V. H., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2 364-2 368 (1972).
- Quastel, M. R., Wright, P., and Kaplan, J. G., *Proc. sixth leucocyte culture conference*, 185-214 (Academic, New York, 1972).
- Novogrodsky, A., *Biochim. biophys. Acta*, **266**, 343-349 (1972).
- Zachowski, A., and Paraf, A., *Biochem. biophys. Res. Commun.*, **57**, 787-792 (1974).

Suppressor T cells in mice made unresponsive to skin allografts

We have established¹ that long-lasting, strain-specific unresponsiveness to H-2 incompatible skin allografts can be induced in 30-50% of adult CBA mice by pretreatment with a single injection of a crude extract of liver from the graft donor strain, combined with a dose of *Bordetella pertussis* vaccine and a short course of antilymphocyte serum (ALS). Lymphoid cells removed from mice bearing healthy well-established allografts were reactive against graft donor strain antigens when tested in mixed lymphocyte culture (MLC), cell-mediated lympholysis tests (CML) and in graft *versus* host assays (GvH)². Haemagglutinating and cytotoxic antibodies specific for the donor strain were invariably absent from the serum of unresponsive mice although small quantities of IgG1 antibodies have been detected by a more sensitive technique³. We have so far been unable to detect any strain-specific suppressive activity in sera using MLC, CML and GvH tests² or when passively transferring sera to skin-grafted mice.

In view of increasing evidence for the existence of suppressor T cells (reviewed in refs 4-6) we have investigated the possibility that they may be important in maintaining the unresponsiveness in our mice. The experiments reported here suggest that they are important in this respect, but it would be premature to infer that suppressor T cells provide the only mechanism for the maintenance of this unresponsiveness.

Spleens were removed from unresponsive CBA mice bearing healthy A strain (= A/Jax) skin grafts 90-200 d after transplantation, and cell suspensions were made in phosphate-buffered Ringer solution with or without 5% foetal calf serum. Spleen cells were injected (half intravenously, half intraperi-

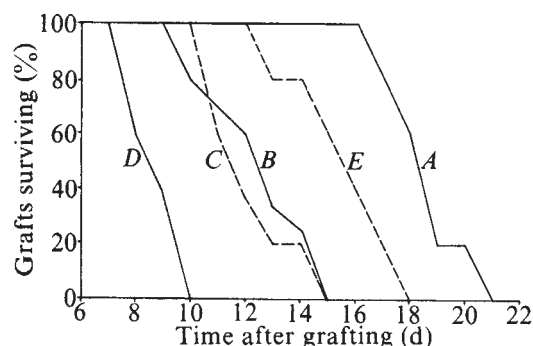


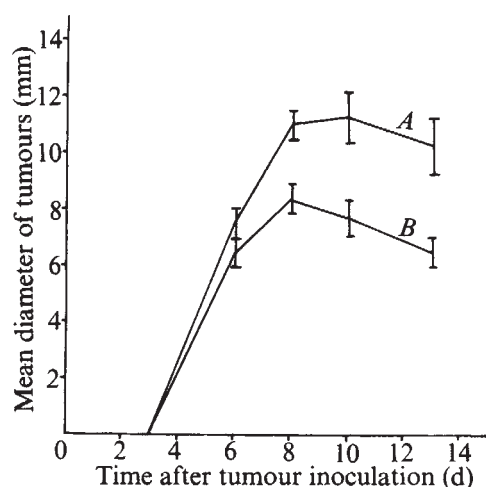
Fig. 1 Adoptive transfer of 2×10^8 unresponsive CBA spleen cells to normal syngeneic mice: effect on survival of A strain skin grafts. Recipients were also treated with serum from their spleen cell donors (see text). A, Unresponsive cells (five mice); B, no treatment (11 mice); C, normal cells (five mice); D, sensitised cells (ten mice); E, unresponsive cells (separate experiment) without serum (five mice).

toneally) into normal or ALS-treated syngeneic recipients which were challenged with A strain skin grafts or injected subcutaneously with A strain sarcoma 1 tumour cells.

Spleen cells (2×10^8) from unresponsive mice were injected into normal CBA males 3 d before grafting with A strain skin. Control mice received skin grafts only or were injected with 2×10^8 spleen cells from normal mice; another group received cells from sensitised mice that had recently rejected A strain skin grafts. In this experiment the mice were treated, in addition, with serum from their spleen cell donors, and were given 0.5 ml intraperitoneally on the day of grafting and 0.2 ml on the following day. Figure 1 shows that the recipients of 'unresponsive' cells (group A) retained their grafts significantly longer than controls (groups B and C). Recipients of sensitised cells (group D) exhibited accelerated rejection. In a separate experiment the adoptive transfer of 2×10^8 spleen cells from unresponsive mice, without additional serum, also prolonged graft survival, though less markedly (group E). We subsequently observed that the passive transfer of as much as 1.5 ml of serum from unresponsive mice, without cells, did not affect the survival of A strain skin grafts in the slightest.

CBA female mice which had been injected with unresponsive spleen cells showed impaired ability to reject A strain sarcoma 1 tumours (Fig. 2). Viable spleen cells (1.4×10^8) from unresponsive or normal mice were injected into CBA females, and on the same day, 10^6 sarcoma 1 cells were inoculated subcutaneously

Fig. 2 Adoptive transfer of 1.4×10^8 unresponsive CBA spleen cells to normal syngeneic mice: effect on growth of A strain sarcoma 1. Tumour growth in mice injected with (A) unresponsive cells (B) normal cells (five mice per group, mean diameters of tumours are plotted $\pm$ s.e.).



into the flank. Figure 2 shows that tumour rejection was significantly suppressed in the recipients of unresponsive cells (group A) compared with controls (group B). Tumours in both groups were eventually rejected but after day 13 ulceration of the tumours prevented precise measurements.

To determine whether T cells were required for the successful transfer of unresponsiveness, spleen cells from unresponsive mice were treated with anti- θ antibodies and complement *in vitro* before transfer. One hundred million viable cells or the same dose reduced to 55×10^6 viable cells by treatment with anti- θ and complement, were injected into CBA male mice which had been grafted with A strain skin 10 d before and which had received a short postoperative course of ALS. Figure 3 shows that the survival of skin grafts was considerably improved by injecting untreated unresponsive cells (groups A and E) or those treated with complement only (group B), whereas pretreatment with anti- θ and complement (group C) abolished this effect and even curtailed graft survival compared with the ALS controls (group E). Normal spleen cells too (group D) hastened rejection.

These experiments and others⁷ suggest that reactivity in mice retaining skin allografts for long periods in this model is under active restraint. Our data provide no direct evidence for the specificity of transferred unresponsiveness, although unresponsiveness in the spleen cell donors was exquisitely strain specific (L.B. and P.J.K., unpublished). That treatment

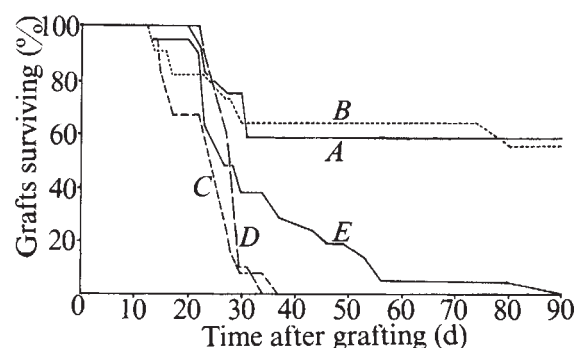


Fig. 3 Treatment of unresponsive CBA spleen cells with anti- θ and complement: effect on the adoptive transfer into ALS-treated secondary hosts of unresponsiveness to A strain skin grafts. ALS (0.5 ml) given on days 1, 3 and 6 after skin grafting, cell transfer on day 10 (10–12 mice per group). A, Untreated unresponsive cells; B, cells treated with complement only; C, cells treated with anti- θ and complement; D, cells from normal mice; E, ALS only.

with anti- θ antibodies and complement abolished suppressor activity from unresponsive spleen cells clearly indicates a role for T cells but it does not distinguish between a direct immunosuppressive effect of T cells and their possible cooperation with B cells (either in the transferred donor population or in the host) in the production of blocking antibodies. The detection of only small quantities of IgG1 antibodies in the serum of spleen cell donors⁸ and the failure of serum to suppress either *in vitro* or *in vivo* reactivity², however, suggest that serum-blocking factors are unlikely to be involved. At present the production of specific suppressor substances directly from T cells⁹ is a more plausible possibility. It is of interest that evidence has recently been presented that suppressor cells may be important in tolerance induced by injection of allogeneic cells into embryos¹⁰ or neonates¹¹.

Confirmation of the data reported here has been found in other transfer experiments using sublethally irradiated as well as ALS-treated secondary recipients (ref. 7 and L.B. and P.J.K., unpublished). We have, however, found considerable variability in the level of unresponsiveness transferred, but purely technical reasons may be responsible.

An anomaly that emerges from our investigation is that

spleen cells from unresponsive mice were reactive against graft antigens in MLC, CML and GvH, but suppressed reactivity against skin grafts when transferred to syngeneic recipients. It is conceivable that the dose of antigen and its presentation may determine whether reactivity or suppression predominates and that the great excess of antigen present in the MLC, CML and GvH assays may circumvent or overwhelm the suppressor mechanism.

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Note added in proof: We now have evidence that purified T cell suspensions can transfer unresponsiveness to ALS-treated recipients. In another experimental model, Diener and his colleagues¹² have demonstrated the presence of suppressor cells in the lymph nodes of mice unresponsive to heart allografts.

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- ¹ Pinto, M., Brent, L., and Thomas, A. V., *Transplantation*, 17, 477-486 (1974).
- ² Brooks, C. G., Brent, L., Kilshaw, P. J., New, R. R. C., and Pinto, M., *Transplantation*, 19, 134-144 (1975).
- ³ Kilshaw, P. J., Brent, L., and Pinto, M., *Transplant. Proc.* (in the press).
- ⁴ Gershon, R. K., in *Contemporary Topics in Immunobiology*, 3, (edit. by Cooper, M. D., and Warner, N. L.), 1-40 (Plenum, New York, 1974).
- ⁵ Droege, W., *Curr. Tit. Immun., Transplant. Allergy*, 1, 95 and 131 (1973).
- ⁶ Playfair, J. H. L., *Clin. exp. Immun.*, 17, 1-18 (1974).
- ⁷ Kilshaw, P. J., Brent, L., Brooks, C. G., New, R. R. C., and Pinto, M., *Transplant. Proc.*, 7, 385-387 (1975).
- ⁸ Feldmann, M., *Eur. J. Immun.*, 4, 667-674 (1974).
- ⁹ Zembala, M., Asherson, G. L., Mayhew, B., and Krejci, J., *Nature*, 253, 72-74 (1975).
- ¹⁰ Rouse, B. T., and Warner, N. L., *J. Immun.*, 113, 904-909 (1974).
- ¹¹ Silvers, W. K., *J. Immun.*, 113, 804-809 (1974).
- ¹² Diener, E., in *Immunological Tolerance* (edit. by Katz, D. H., and Benacerraf, B.), 543 (Academic, London, 1974).

Functional fractionation of human cytotoxic cells using differences in their cation requirements

DEMONSTRATION of anti-tumour immunity in man is often impaired by the variable level of cytotoxicity of normal control cell populations. Using physical fractionation techniques, attempts have been made to remove selectively the cells which are responsible for this 'variable background' effect^{1,2}, and to improve characterisation of cytotoxic cell populations. We report here that several types of human cell-mediated cytotoxicity have different extracellular cation requirements either for Ca^{2+} , for Mg^{2+} , for both, or for neither. These findings suggest the existence of differences in the mechanism(s) of lysis, and lead us to propose an alternative, functional approach to the fractionation of cytotoxic cells.

Four different systems were investigated with human

peripheral blood cells as effector cells: anti-allogeneically sensitised cells (thymus-derived, T, cytotoxic cells³) against lymphocytes transformed with phytohaemagglutinin (PHA blasts); normal cells against either antibody-coated erythrocytes or antibody-coated tumour cells (non-T cytotoxic cells in both instances^{3,4}); uncoated tumour cells.

Cytotoxicity of PHA blasts by *in vitro* sensitised anti-allogeneic human cells required Ca^{2+} (Fig. 1c). This Ca^{2+} dependence involved both the nonspecific (on a syngeneic target cell) and the specific components of cytotoxicity, and was similar to the predominant Ca^{2+} dependence of cytotoxicity mediated by mouse T cells (refs 5-7 and P. G. and E. Smith, unpublished). Other experiments showed that, although Mg^{2+} alone could not support cytotoxicity, it tended to increase the cytotoxicity obtained in the presence of Ca^{2+} .

Lysis of antibody-coated sheep red blood cells (SRBC-Ab) by unsensitised human blood cells could occur in the absence of added cations, and was increased by the addition of Mg^{2+} more than by the addition of Ca^{2+} (Fig. 1b). This was found in each of five experiments, which also showed the absence of toxicity of effector cells for uncoated (control) SRBC. The predominant Mg^{2+} requirement of this system in human and also mouse⁸ effector cells contrasts markedly with the cation requirements of the other cytotoxic systems described here. Moreover, the cytotoxicity for SRBC-Ab observed in the absence of added cations was not entirely accounted for by cation contamination, because it was not abolished by the addition of an excess of EDTA (Table 1). Thus, lysis of SRBC-Ab by human cells has two components, one of which is predominantly Mg^{2+} -dependent and the other which cannot be blocked by EDTA and is therefore divalent cation-independent. Lysis of antibody-coated erythrocytes by normal mouse spleen cells is Mg^{2+} -dependent⁸, whereas their lysis by normal mouse peritoneal cells cannot be blocked by EDTA⁹. A likely interpretation of these results is that two functionally different effector cell populations against antibody-coated erythrocytes, detected in different anatomical locations in mouse, occur in human blood. Note that we are probably dealing here with extracellular lysis, not phagocytosis, as the rate of ^{51}Cr release would be much slower in the latter case¹⁰ and phagocytosis is inhibitable by EDTA⁹.

Lysis of antibody-coated Chang human liver tumour cells (Chang-Ab) by unsensitised human blood cells was predominantly Ca^{2+} -dependent (Fig. 1a). This was found in each of six experiments. In some of these, it was found in addition that Ca^{2+} and Mg^{2+} together may support a somewhat higher level of cytotoxicity than Ca^{2+} alone (Table 2). This effect was linked, at least in part, to the 'spontaneous' cytotoxicity, at high ratios, of normal human cells towards uncoated Chang cells. The level of this spontaneous cytotoxicity varied from individual to individual, from very marked to minimal. Note that it was substantial only in the presence of both Ca^{2+} and Mg^{2+} (Table 2). A time course experiment (Fig. 2) also showed that it was not the absolute concentration of any divalent

Table 1 Cytotoxicity (percentage ^{51}Cr release) of human peripheral blood cells for antibody-coated SRBC, in the absence or presence of Ca^{2+} and/or Mg^{2+} , with or without EDTA

Experiment	Cytotoxic cells*	EDTA (2 mM)	Ca^{2+} (1 mM)	Mg^{2+} (1 mM)	Ca^{2+} (0.5mM) + Mg^{2+} (0.5mM)	No added cations
1	Human blood cells	—	34	41	50	23
	None	—	6	6	6	6
	Human blood cells	+	16	15	16	16
	None	+	6	6	6	6
2	Human blood cells	—	34	39	49	27
	None	—	7	6	7	7
	Human blood cells	+	22	21	22	22
	None	+	9	9	8	9

*Normal peripheral blood cells, prepared as described in Fig. 1, tested in a 4 h ^{51}Cr release test at a ratio of 1:1 target cell, in the presence of the indicated concentration of cations and/or EDTA.

Table 2 Cytotoxicity of human peripheral blood cells for Chang cells, uncoated or coated with antibody, in the presence of Ca^{2+} and/or Mg^{2+}

Experiment*	Target cells	Ca^{2+} (1 mM)				Mg^{2+} (1 mM)				Ca^{2+} (1 mM) + Mg^{2+} (1 mM)			
		(40)†	(20)	(10)	(0)	(40)	(20)	(10)	(0)	(40)	(20)	(10)	(0)
1	Chang	4	5	3	0	2	1	2	0	23	19	17	2
	Chang-Ab	38	37	31	1	18	10	10	4	42	39	32	5
2	Chang	1	2	0	0	1	1	0	0	6	4	3	1
	Chang-Ab	22	18	11	2	7	6	7	5	26	20	14	4
3	Chang	5	2	3	0	3	2	2	3	8	8	4	3
	Chang-Ab	13	10	1	-4	-2	-3	-2	-3	24	17	8	-3
4	Chang	-3	-8	0	0	1	0	1	1	8	7	7	4
	Chang-Ab												

*Peripheral blood cells, prepared as described in Fig. 1, taken from four different apparently normal subjects, were tested in a 4 h ^{51}Cr release test against Chang cells, with or without rabbit anti-Chang antiserum, in the presence of the indicated concentration of cations. Cytotoxicity is expressed as percentage ^{51}Cr release minus spontaneous ^{51}Cr release in the presence of Ca^{2+} alone.

†Different ratios of effector to target cells.

cation, but the actual presence of both Ca^{2+} and Mg^{2+} which was important. Preliminary experiments indicate that the toxicity of normal human blood cells over short incubation periods is largely Ca^{2+} plus Mg^{2+} -dependent, not only in the case of Chang cells, but also for other human tumour cell lines.

We found that different types of human cell-mediated cytotoxicity have different cation requirements. Lysis of PHA blasts mediated by T cells requires Ca^{2+} , lysis of SRBC-Ab mediated by non-T cells preferentially requires Mg^{2+} or no

cations, lysis of Chang-Ab mediated by non-T cells requires Ca^{2+} , and the anti-Chang spontaneous cytotoxicity requires both Ca^{2+} and Mg^{2+} . Note that these differences in cation requirements are relative. For instance, in every system requiring either Ca^{2+} or Mg^{2+} , the 'non-required' cation, poorly effective by itself, tends to increase the cytotoxicity obtained in the presence of the 'required' cation. The anti-Chang system may represent an extreme expression of the same phenomenon. Also, the results suggest that the differences in cation requirements are not exclusively linked to the nature of

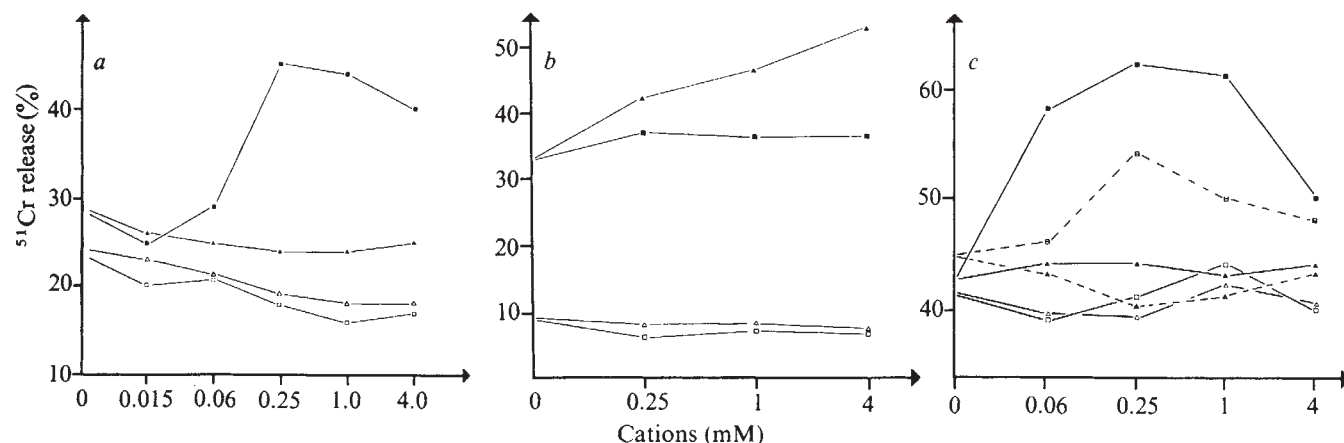


Fig. 1 Cytotoxicity (expressed as ^{51}Cr release) of human cells for various types of target cells, in the presence of graded concentrations of Ca^{2+} ($\square$, $\blacksquare$) or Mg^{2+} ($\triangle$, $\blacktriangle$). Cytotoxicity was measured using a 4 h ^{51}Cr release test, after the addition of known amounts of Ca^{2+} and/or Mg^{2+} to systems initially as free as possible of divalent cations. Three measures were taken to reduce unwanted cation contamination. First, we used only disposable plastic material, not washed glassware. Second, both cytotoxic and target cell populations were pretreated with Versene (1.5 mM EDTA solution). Third, the medium used for all subsequent steps, including the cytotoxicity test, was a modified Eagle's medium specially prepared without CaCl_2 and MgCl_2 (ICRFL) plus 1% heat-inactivated foetal calf serum (FCS) dialysed for 48 h against double-distilled water. This percentage of FCS was chosen in preliminary experiments as a compromise between lower percentages (detrimental to lysis mediated by T cells) and higher percentages (blocking lysis mediated by non-T cells of antibody-coated erythrocytes). As it is, this medium could be used for all types of cell-mediated cytotoxicity described in this report. Its contamination by divalent cations, repeatedly checked by atomic absorption spectrophotometry, averaged 0.036 mM and 0.010 mM for Ca^{2+} and Mg^{2+} , respectively. Human peripheral blood cells, sensitised or not, were incubated in a Versene solution for 30 min at 37°C , washed twice, adjusted to the required concentration and distributed in V-shaped wells of IS-MVC-96 Linbro microplates. Each well also received target cells (5×10^3 PHA blasts or 10^5 SRBC or 10^4 Chang cells, labelled overnight with ^{51}Cr as previously described¹⁵, washed once with Versene and twice with medium), antiserum when required (rabbit anti-Chang or rabbit anti-SRBC antisera, at a final concentration of 1:10,000) and solutions of CaCl_2 or MgCl_2 to a total volume of 0.02 ml. The microplates were centrifuged (280g, 2 min) to increase the sensitivity of the cytotoxicity test^{15,16}, incubated for 4 h at 37°C in a humidified CO_2 incubator and centrifuged again. Volumes (0.1 ml) of supernatants were sampled with an Eppendorf micropipette, their radioactivity measured and expressed as percentage total radioactivity per well. In this type of assay, an experimental difference of 5% or more is statistically significant ($P = 0.05$). a, Cytotoxicity of normal human cells obtained by Ficoll-Triosil fractionation of defibrinated blood, at a ratio of 10:1 Chang-Ab ($\blacksquare$, $\blacktriangle$). $\square$, $\triangle$, Spontaneous release by Chang-Ab alone. b, Cytotoxicity of normal human cells obtained by Ficoll-Triosil fractionation of heparinised blood, at a ratio of 1:1 SRBC-Ab ($\blacksquare$, $\blacktriangle$). $\square$, $\triangle$, Spontaneous release by SRBC-Ab alone. c, Cytotoxicity of sensitized cells for PHA blasts. Normal human cells were obtained by Ficoll-Triosil fractionation of defibrinated blood of subjects A and B. A anti-B and B anti-A sensitized cells were obtained after 6 d of cocubation at 37°C in a 10% CO_2 atmosphere of 2×10^6 responding human cells and 2×10^6 irradiated (2,000 rad) stimulating human cells, in 2 ml of modified Eagle's medium plus glutamine and 10% human serum, per well of FB-16-24-TC Linbro plates. Either A or B target PHA blasts were obtained by incubating normal cells for 3 d in the presence of PHA at a concentration of 1:100. For the cytotoxicity test, the effector-target cell ratio was 15:1. If target cells are PHA blasts obtained from A cells; $\blacksquare$, $\blacktriangle$, effect of B anti-A; $--\square--$, $--\triangle--$, effect of A anti-B sensitized cells; $\square$, $\triangle$, spontaneous release by PHA blasts alone.

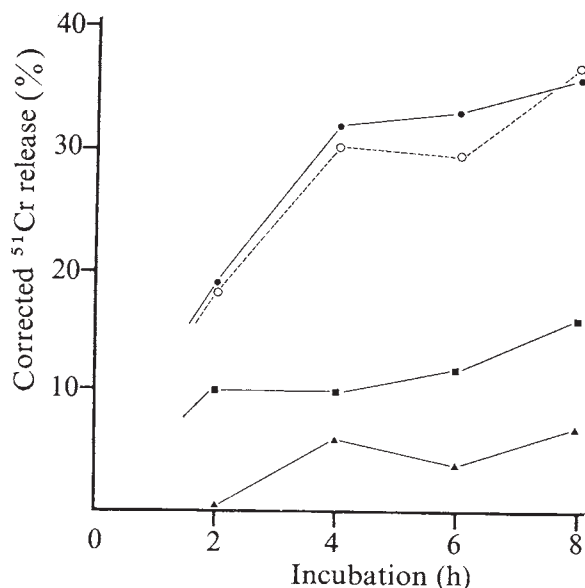


Fig. 2 Cytotoxicity of normal human peripheral blood cells for Chang cells, expressed as corrected ^{51}Cr release (^{51}Cr release minus spontaneous ^{51}Cr release by target cells alone), at a ratio of 100 effector cells to 1 target cell, as a function of time and of the presence of either 1 mM Ca^{2+} (■), or 1 mM Mg^{2+} (▲), or 1 mM Ca^{2+} plus 1 mM Mg^{2+} (●), or 0.5 mM Ca^{2+} plus 0.5 mM Mg^{2+} (○).

the target cells. These findings have implications for both the mechanism of lysis and the nature and fractionation of cytotoxic cells. As for the mechanism of lysis, the difference in cation requirements described here do not seem to apply to the recognition step of cytolysis (compare with refs 11–14) and thus may well reflect differences between the actual post recognition lytic mechanisms of various systems (P. G. and E. Smith, unpublished); and the results obtained in the anti SRBC–Ab system reinforce our previous conclusion⁸ against a conventional, extracellular Ca^{2+} -dependent, 'stimulus–secretion' mechanism in this type of cytolysis. The potential of a functional fractionation of cytotoxic cells on the basis of their cation requirements is demonstrated by the specification of the individual characteristics of the cytotoxic activity of normal human blood cells against antibody-coated erythrocytes from other cytolytic systems, and its further dissection into two functional components. Furthermore, when tumour cells are used as targets, our results suggest that Mg^{2+} -free conditions may depress the cytotoxicity of normal control cell populations more than specific anti-tumour cytotoxicity, which would be an obvious advantage in anti-tumour research in man.

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¹ Jondal, M., and Pross, H., *Int. J. Cancer* (in the press).

² Peter, H. H. et al., *J. Immun.* (in the press).

³ Chess, L., MacDermott, R. P., Sondel, P. M., and Schlossman, S. F., *Progr. Immun.*, 2, 125–132, 1974.

⁴ Perlmann, P., Perlmann, H., and Wigzell, H., *Transplant. Rev.*, 13, 91 (1972).

⁵ Maue, J., Rudolf, H., Chapuis, B., and Brunner, K. T., *Immunology*, 18, 517–535, (1970).

⁶ Henney, C. S., and Bubbers, J. E., *J. Immun.*, 110, 63–72 (1973).

⁷ Martz, E., and Benacerraf, B., *J. Immun.*, 111, 1538–1545 (1973).

⁸ Golstein, P., and Gomperts, B. D., *J. Immun.*, 114, 1264–1269 (1975).

⁹ Scornik, J. C., and Cosenza, H., *J. Immun.*, 113, 1527–1532 (1974).

¹⁰ Hersey, P., *Transplantation*, 15, 282–290 (1973).

¹¹ Stalling, R. D., and Berke, G., *J. exp. Med.*, 137, 932–942 (1973).

¹² Scornik, J. C., *J. Immun.*, 113, 1519–1526 (1974).

¹³ Berken, A., and Benacerraf, B., *J. exp. Med.*, 123, 119–144 (1966).

¹⁴ Lay, W. H., and Nussenzweig, V., *J. Immun.*, 102, 1172–1178 (1969).

¹⁵ Golstein, P., and Blomgren, H., *Cell. Immun.*, 9, 127–141 (1973).

¹⁶ Thorn, R. M., Palmer, J. C., and Manson, L. A., *J. Immun. Meth.*, 4, 301–315 (1974).

Radiation response of C3H fibrosarcoma enhanced in mice stimulated by *Corynebacterium parvum*

TREATMENT of the laboratory mouse with *Corynebacterium parvum* can potentiate efficacy of host reaction against tumours^{1–4}. This has been reflected in the proportion of mice whose tumours: (1) grew more slowly; (2) regressed partially before continuing growth, and (3) regressed completely, either temporarily or permanently. Furthermore, in some mouse tumour systems chemotherapy is more effective when hosts are stimulated with *C. parvum*. We have now found that administration of *C. parvum* before local irradiation of 8-mm diameter fibrosarcoma isografts increases the frequency of cure, strikingly so after low radiation doses.

A C3H mouse fibrosarcoma (induced by methylcholanthrene) was used as sixth generation isografts growing in the right legs of C3Hf/Sed mice (bred and maintained in our colony which is pathogen-free and carries a defined flora)⁵. The fibrosarcoma has been shown to be immunogenic by quantitative cell transplantation studies in normal, tumour-immunised, and immunosuppressed recipients⁶. Treatment with *C. parvum* began 7–9 d after transplantation when the tumours were 5 mm in diameter. We injected 0.35 mg intravenously (0.4 ml) or intralesionally (0.1 ml) of the suspension was deposited along six needle tracks around and through the tumour). When the tumours reached 8 mm in diameter, ^{137}Cs radiation was given as a single dose at 950 rad min⁻¹. Radiation technique was based on a 3-cm diameter treatment portal so that only the tumour and the affected limb were irradiated. For irradiation, the mice were taped to a restraining plate in a 250-ml chamber, sealed and ventilated with 2 l of sterile air per min. Results have been tabulated as the proportion of mice free of tumour 80 d after treatment.

Table 1 shows that up to 60% of mice were cured permanently of their 5-mm diameter tumour by *C. parvum* alone. In successfully treated mice, tumour growth did not differ from that in control mice for 5–10 d after injection of *C. parvum*; regression then commenced and was usually complete within another 10 d. Regression was more likely if *C. parvum* was administered both intralesionally and intravenously than by either route alone; the sequence of injection routes did not seem important. The remaining data demonstrate a significant increase in tumour control after local irradiation in mice pretreated with *C. parvum*. The efficacy of *C. parvum* was not demonstrably greater when it was administered intravenously and intralesionally when the tumour was also subjected to local irradiation. The increased efficacy of administering *C. parvum* and local irradiation was most impressive for small doses of radiation. The difference in frequency of tumour destruction between control and *C. parvum* treated animals whose tumours received 800 or 1,500 rad was significant at $P \leq 0.01$.

The difference in the probability of survival between the mice given radiation alone and those given *C. parvum* and radiation was 0.68 with low doses (800–1,500 rad) and 0.11 with higher doses (2,250–3,750 rad); the difference between these two values is significant ($P = 0.007$). (χ^2 test for heterogeneity). This finding that *C. parvum* potentiates the efficacy of radiation administered at low but not at high doses, in this tumour system, emphasises the need for information about the role of radiation-induced changes in tumours and in adjacent normal tissue if the efficacy of the host's attempt to reject a

Table 1 Proportion of mice free of tumour 80 d after irradiation

<i>C. parvum</i>	0	200	400	800	1,500	2,250	3,000	3,750
None	0/20		0/5	0/10	2/10	2/5	4/9	14/15
Intravenous								
5-mm tumour	10/29	8/10	8/10	10/14	13/15	9/15	9/10	11/13
Intralesional								
5-mm tumour	2/10			0/4	1/5	4/5	4/5	4/5
Intravenous								
5-mm tumour	11/20	8/10	10/10	12/15	5/5	2/4	4/5	4/4
Intralesional								
8-mm tumour								
Intralesional								
5-mm tumour	6/10			3/4	4/5	3/5	4/4	4/5
Intravenous								
8-mm tumour								

Figures show radiation dose-tumour control response data for 8-mm diameter fibrosarcoma (isotransplants in right leg) alone or combined with *C. parvum*.

tumour graft is to be modified. Although the precise mechanism of action of *C. parvum* is not known, the observation of such a powerful adjuvant effect with local irradiation may have potential interest for radiation therapy.

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- ¹ Halpern, B. N., Biozzi, G., Stiffel, C., and Mouton, D., *Nature*, **212**, 853-854 (1966).
- ² Woodruff, M. F. A., and Boak, J. L., *Br. J. Cancer*, **20**, 345-355 (1966).
- ³ Milas, L., and Mujagic, H., *Eur. J. clin. biol. Res.*, **17**, 498-500 (1972).
- ⁴ Fisher, J. C., Grace, W. R., and Mannick, J. A., *Cancer*, **26**, 1379-1382 (1970).
- ⁵ Amiel, J. L., and Bernardet, M., *Eur. J. Cancer*, **6**, 557-559 (1970).
- ⁶ Currie, G. A., and Bagshawe, K. D., *Br. med. J.*, **1**, 541-544 (1970).
- ⁷ Pearson, J. W., Pearson, G. R., Gibson, W. T., Chermann, J. C., and Chirigos, M. A., *Cancer Res.*, **32**, 904-907 (1972).
- ⁸ Jurin, M., and Suit, H., *Cancer Res.*, **32**, 2201-2211 (1972).

Drug-induced phase change in bilayer as possible mode of action of membrane expanding drugs

MANY small molecules modulate membrane functions. Substances such as neurotransmitters interact directly with specific protein receptor sites, whereas others such as anaesthetics, interact with membrane lipids or with hydrophobic regions of membrane. This is consistent with a correlation of their activity with their lipid solubility and lipid-water partition coefficient¹. Several lipid-soluble drugs are antihemolytic², which correlates not only with their pharmacological activity but also with their ability to expand the lipid bilayer of a biomembrane³. Although expansion of bilayers implies a drug-induced reorganisation of lipids, the mechanism involved remains to be established. We now have evidence that the lipid-soluble drugs may induce a transition of lipid acyl chains from organised gel to randomised liquid crystalline phase. Such drug-induced changes in the lipid phase in a bilayer may affect the function of various membrane-bound proteins⁴.

The effect of drugs on phase transition behaviour of mechanically dispersed dipalmitoyl lecithin (DPL) liposomes was studied by differential scanning calorimetry. Samples

for scanning contained 75 mM DPL, 50 mM KCl, 5 mM Tris-HCl and the appropriate concentration of drug (0.1-10 mM) at pH 7.4. After mixing, the samples were allowed to equilibrate for 2-3 d at 4 °C before they were scanned on DSC-1B (Perkin Elmer) in sealed sample pans. For all runs a sensitivity of 1 mcalorie s⁻¹ was used with a scanning rate of 1.25 K min⁻¹ between 295 and 320 K. The enthalpies of transition were calculated from the areas under the curves, which were determined by weighing the traces of the transition profiles.

The transition profile for DPC dispersion without any drug showed a transition temperature at 314.6 K with enthalpy of the transition +8.9 kcalorie mol⁻¹, and the width and half-height width for the transition as 1.6 K and 0.60 K respectively. In the liposomes which contained a drug,

Table 1 Concentrations of various drugs which double the width at half-height of the transition profile

Drug	Conc. (mM)	Type of transition profile*
Alkanols		
n-Pentanol	23	A
n-Hexanol	5.1	A
n-Heptanol	2.5	A
n-Octanol	1.14	A
Local anaesthetics		
Procaine	17	A
Nesacaine	15	A
Benzocaine	3.5	A
Butacaine	3.0	A
Tetracaine	3.3	A
Dibucaine	1.8	A/B
Xylocaine	6.7	A
Marcaine	2.4	A
Uncouplers		
TTMB	3.3	B
CCP	7.5	B
Cl-CCP	5.3	B
FCCP	4.4	B
Tranquillisers		
Prochlorperazine	0.7	A
Trifluoroperazine	1.6	A
Trimiprazine	5.2	A
Chlorpromazine	4.5	A
Inhalation anaesthetics		
Diethyl ether	85	C
Chloroform	10	C
Halothane	3.1	A/C
Fluroxane	4.5	C
Enflurane	25.5	C

* TTMB (3,4,5,6-tetrachloro-2-trifluoromethylbenzimidazole); FCCP, (carbonylcyanide-*p*-trifluoromethoxy phenylhydrazide); CCP, (carbonylcyanide phenylhydrazide); Cl-CCP, (*m*-chloro-CCP). For type B profiles, the concentration given reduced the area under the main peak by 50%. For type C profiles, the concentration given shifted the peak at half-height by 0.6 K. Some drugs, like dibucaine and halothane, showed two types of profile. The first at low concentrations and the second at higher concentrations.

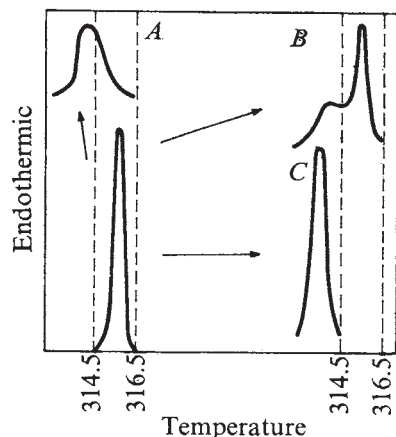


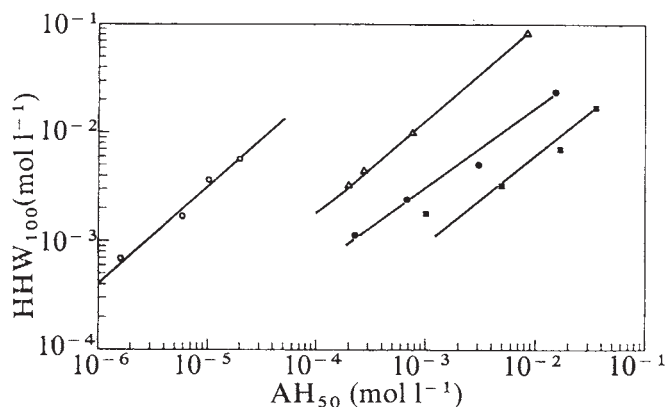
Fig. 1 Typical DSC transition profiles for DPL liposomes doped with various drugs. Three types of profiles have been observed for the drugs shown in Table 1. In type *A*, an asymmetrical broadening of the main peak is observed. This broadening is accompanied by an increase in the half-height width and a decrease in T_c . In type *B* profiles, the area of the main peak decreases, and the area of the shoulder at lower temperatures increases with increasing drug concentration. T_c for the main peak seems to remain unchanged, whereas T_c for the shoulder decreases at higher drug concentrations. In type *C* profiles the main peak shifts to lower temperatures at successively higher drug concentrations. No broadening of the peak is observed. The total area under the curves (enthalpy of transition) seems to remain constant in all these profiles. In the temperature range for transition, several phases may coexist within the plane of the bilayer.

the transition profile was characteristic for the type of drug. In general, three distinct types of transition profile (Fig. 1) were obtained for the drugs examined (Table 1). Thus all uncouplers of oxidative phosphorylation showed type *B*, local anaesthetics showed type *A*, and inhalation anaesthetics showed type *C* transition profiles. For all drugs, a broadening of the transition profile accompanied a lowering in the phase transition temperature (T_c) of DPL.

For most of these doped bilayers, however, there was little or no change in the enthalpy of transition. The effect of various drugs on half-height width (type *A*), relative area (type *B*) or shift (type *C*) of the main transition peak was linearly concentration-dependent and did not seem to show a saturation behaviour in the range (up to 10 mM for most drugs) we examined.

These effects on transition profiles therefore relate to

Fig. 2 The correlation between the concentrations of drugs that increase the half-height width by 100% (HHW_{100} ; data from Table 1) and concentrations of the same drugs which protect human erythrocytes by 50% against haemolysis (AH_{50} ; data from ref. 2). For inhalation anaesthetics the abscissa is AH_8 (8% protection; data from ref. 3) and ordinate is the concentration at which the transition peak shifts by 0.6 K at half height. ●, *n*-Alkanols; ■, local anaesthetics; ○, phenothiazine tranquilisers; △, inhalent anaesthetics.



the fraction of the bilayer modified by the drug. Regardless of the mechanism of incorporation of the drugs in bilayers, their effects on transition profile seem to depend on their concentration in the liposome. Thus the effects on transition profiles may be correlated with other concentration-dependent effects on membrane, such as antihemolytic activity. As Fig. 2 shows for a series of drugs, the concentration that induces a certain effect on the liposome bilayer (Table 1) is related to the corresponding concentrations for 50% or 8% antihemolysis of human red cells². Correlation for each drug type is good. Variation between the drug types is quite large. All the lines seem, however, to be parallel. This variation may imply a difference in the partition coefficient for these drug types in DPL liposomes and human erythrocytes. Only dibucaine, which has a structure different from other anaesthetics, does not fall on the line for local anaesthetics.

The transition from gel to liquid crystalline phase in a phospholipid bilayer can be described as a highly cooperative transition from order to disorder, involving a lateral expansion and a decrease in thickness and density^{7,8}. Within the transition range (Fig. 1), two-dimensional domains of fluid and gel phase coexist. Below and above this range, the membrane exists in gel and fluid phases respectively. The data presented here show that addition of lipid-soluble drugs not only lowers the transition temperature but also enlarges the temperature range in which two-dimensional domains of fluid and gel phases coexist. Therefore, in the presence of drugs at a given temperature a greater fraction of membrane is in the fluid phase. These results support the conclusion that anaesthetics and related lipid-soluble drugs cause an increase in fluidity, disorder and mobility of the lipid bilayer⁸⁻¹⁰ which can modulate the function of membrane proteins^{4,11}.

These observations correlate the pharmacological activity and membrane expanding ability of various drugs to their ability to lower the gel to liquid crystalline transition temperature in lecithin bilayers (Fig. 2). A rationale for this correlation can be seen in the suggestion that within an optimum temperature range the domains of gel and liquid crystalline state may coexist in the bilayer of biomembranes¹². A drug-induced decrease in the transition temperature would imply that in biomembranes small molecules can induce effects similar to those elicited by increasing temperature in a critical range. This hypothesis is also consistent with the observation that the volume expansion of membrane is about ten times the bulk volume of the drug² that induces it. Drugs may also affect the proportion of lipid in the two or more phases which coexist within a bilayer. Consequently, a drug-induced increase in membrane fluidity would result largely from a simultaneous increase in the fluid and a decrease in the gel region.

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- Hansch, C., and Dunn, N. J., *J. Pharm. Sci.*, **61**, 1-19 (1972).
- Roth, S., and Seeman, P., *Nature new Biol.*, **231**, 284-285 (1971).
- Seeman, P., *Pharmac. Rev.*, **24**, 583-655 (1972).
- Fourcans, B., and Jain, M. K., *Adv. Lipid Res.*, **12**, 147-226 (1974).
- Oldfield, E., and Chapman, D., *FEBS Lett.*, **23**, 285-297 (1972).
- Oldfield, E., *Science*, **180**, 982-983 (1973).
- Rothman, J. E., *J. Theor. Biol.*, **38**, 1-16 (1973).
- Trudell, J. R., Payan, D. G., Chin, J. H., and Cohen, E. N., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 210-213 (1975).

- ⁹ Metcalfe, J. C., Seeman, P., and Burgen, A. S. V., *Molec. Pharmacol.*, **4**, 87-95 (1968).
¹⁰ Hubbell, W. C., McConnell, H. M., and Metcalfe, J. C., *Br. J. Pharmacol.*, **35**, 374-375 (1969).
¹¹ Sullivan, K., Jain, M. K., and Koch, A., *Biochim. biophys. Acta.*, **352**, 287-297 (1974).
¹² Shimshick, E. J., and McConnell, H. M., *Biochemistry*, **12**, 2351-2360 (1973).

Voltage distribution across nerve membranes

EXPERIMENTS on nerve membranes and model systems (such as black lipid films modified by excitability-inducing material) often show conductance parameters which seem to be closely related to Maxwell-Boltzmann statistics¹. There are, however, some difficulties when trying to fit simple Maxwell-Boltzmann factors to experiments. One often has to introduce an effective charge that is not an integer times the electronic charge and often the effective charge changes with voltage. We show here that the effective charge and its nonlinearity may be a consequence of the voltage distribution across the membrane. This implies that the so-called gating particles² may be univalent charges—for example, electrons (I.L. and M.S., unpublished).

By assuming that the active areas in a membrane are surrounded by polar regions that are polarisable, that is, the dipole moments change with electric field we show that the voltage drop (V_a) over the portion of membrane inside the polar regions can be larger than the total membrane voltage V .

The exact calculation of the behaviour of these polarisable dipole layers in the presence of a potential difference between the inside and outside of the membrane is a rather difficult task involving the interplay between electrical and molecular forces, and the nature and distribution of the dipoles. Furthermore, if the dipoles are located mainly at the conducting channels our problem is two or three-dimensional and not one-dimensional as in ordinary calculations of membrane potential distributions.

A simple qualitative illustration is given in Fig. 1. The voltage distribution along an ionic channel is shown for different voltage differences, V , across the membrane. The presence of polarisable dipole layers is seen to change the voltage across the active region, V_a , more than the change in $V - V_0$ (Fig. 1), where V_0 is the voltage on the outside of the membrane. This is caused by a change in the voltage drop across the dipole layers. The voltage drop across a dipole layer is, in principle, given by the number of dipoles, their density and their moment, that is the distance (in the x direction) between the positive and negative charge. For polarisable dipoles this distance is determined by a balance between electrical and mechanical (molecular) forces working on the charges in the dipoles (Fig. 1).

In a simple one-dimensional model the dipoles are regarded as a positive and negative sheet charge separated by a distance, x . We obtain

$$V_a = (V - V_0) [1 - (2\epsilon_a \sigma^2 / \epsilon^2 d \cdot K)] \quad (1)$$

$$= (V - V_0) / (1 - \gamma)$$

for the special case of identical dipole layers at the outside and inside of the membrane, neglecting any extraneous surface charge and any change in voltage drop across the Gouy-Chapman layers. σ is the density of positive (and negative) charge, ϵ_a and d the dielectric permittivity and thickness of the active region, respectively (Fig. 1). ϵ_a is the dielectric permittivity of the material in the dipole layer, K is a force constant (N/m^3) describing the mechanical (molecular) forces acting on the charges in the dipoles. Equation (1) was derived assuming $x \ll d$. The change in equilibrium distance, x , between the positive and negative charges in the dipoles associated with equation (1) is

$$\Delta x = \pm (\epsilon_a \gamma / 2\sigma(1 - \gamma)) (V - V_0) \quad (2)$$

($\gamma < 1$ is a necessary condition for a stable system with minimum

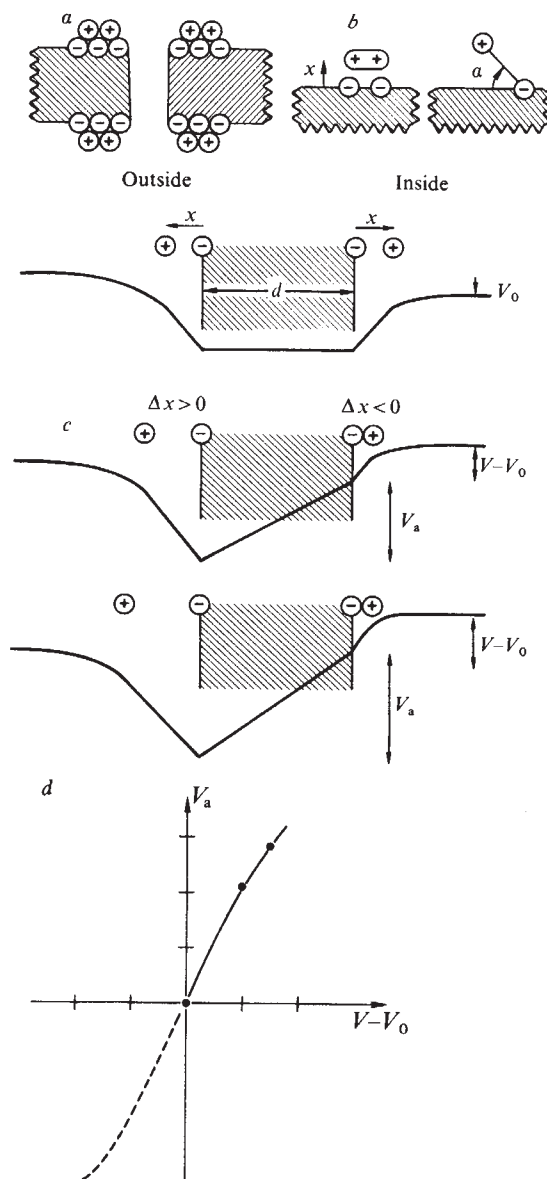


Fig. 1 Schematic illustration of the influence of polarisable dipoles on the voltage distribution across an ionic channel. *a*, Channel with dipoles at the outside and inside of the membrane wall. More negative than positive charges are shown to illustrate the presence of fixed negative charges at the membrane wall. *b*, Two hypothetical types of dipoles; positive charge (in this case a divalent ion) adsorbed to the negative charge at the channel and a permanent dipole at the channel. In both cases it is assumed that the distance between the positive charge and the membrane wall depends on the electric field perpendicular to the wall. The value of x or a is determined by a balance between electrical forces and molecular forces acting on the charges. In our simple model the negative charge is assumed to be fixed. *c*, Voltage distribution along an ionic channel for different voltage differences V between the outside and the inside of the membrane. *d* Is the length of a channel inside the polar region. The change in equilibrium position between the positive and negative charge in the dipole is made very large for illustrative purposes. In reality $|\Delta x|$ will be small compared with the membrane thickness. The last picture illustrates the fact that when the charges get close to each other very small changes in x can be expected because of large repulsive forces. It is seen that $V_a > V - V_0$ because of the change in the voltage drop across the dipole layer. Voltage drops across the Gouy-Chapman layers indicated in the drawing are assumed to be approximately constant. *d*, V_a versus $V - V_0$ obtained for the model presented in (c). Dots correspond to the different drawings in (c). Negative part of the curve can be constructed in a similar way. Because of the properties of the dipole layers on the outside and inside (one may even be missing), V_a as a function of $V - V_0$ may be asymmetric around $V - V_0 = 0$.

total energy.) Experiments on nerve axons and model systems indicate that the gating particle has (or the gating process behaves as) an apparent valence which (1) varies with V and (2) has values varying between say 1 and 5. It is evident that an explanation may be found in the voltage distribution across the membrane instead, as equation (1) implies that a particle with charge q will behave like a particle with charge $q/(1-\gamma)$ related to the applied voltage V .

It is seen that dipoles with large enough polarisability (small enough K) will give a γ of sufficient magnitude to account for point (2) above. $\gamma=0.5$, which corresponds to $V_a=2(V-V_o)$, requires for example $\Delta x=\pm 0.0025(V-V_o)$ Å for $V-V_o$ in mV if $\sigma=10^{18}$ electronic charges per m² and $\epsilon_a=\epsilon_d=10\epsilon_o$. Small (field-dependent) displacements of the charges in the dipole layers thus give a considerable dependence of V_a on $V-V_o$.

Point (1) above, which is equivalent to a nonlinear dependence of V_a on $V-V_o$, is easy to understand in the present model. When the positive and negative charges are pushed close to each other the intermolecular repulsion forces (as a result of overlap of electronic orbitals, for example) are increased rapidly giving rise to a smaller Δx for a given change in $V-V_o$ (to a larger force constant K in equation (1)). The result will be a nonlinear V_a versus $V-V_o$ (Fig. 1).

Thus, to obtain a nonlinear relationship between the active voltage V_a and the applied voltage V with slope >1 we need dipoles with a suitable polarisability close to the ionic channels. We are not restricted to the situation shown in Fig. 1. Dipoles may be present only on one side of the membrane. It is also possible that dipole layers with opposite polarity may be present on the same side of the membrane. The necessary dipoles may be an integral part of the channel molecules or their closest surroundings. These dipoles may also consist of fixed negative and adsorbed positive charges. These two possibilities agree with other theories and experiments on nerve membranes and model membrane systems^{3,4}.

A necessary consequence of the present model is that the voltage distribution across the membrane wall (in the region of an ionic channel) is largely determined by the dipole layers. A general property of the model is that the larger the polarisability is, the smaller K is, the larger will be the parameter γ .

The most important conclusion from the above is that the nonlinearity of nerve membranes and model systems may be explained by the relationship of the voltage drop across the active parts of the membrane and the externally applied voltage. It is not necessarily explained by gating particles with a large electronic charge or by the cooperative movement of a number of charged entities. In a recently proposed electronic model for nerve membrane excitability we used this result (I.L. and M.S., unpublished). The Hodgkin-Huxley parameters were satisfactorily reproduced using a slightly nonlinear V_a against V relationship with $\gamma_{\max} \approx 0.5$. A more detailed calculation on the presented model will be given elsewhere.

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¹ *Biophysics and Physiology of Excitable Membranes* (edit. by Adelman, W. J., Jr) (van Nostrand Reinhold, New York, 1971).

² Armstrong, C. M., and Bezanilla, F., *Nature*, **242**, 459-461 (1973).

³ Ehrenstein, G., Lecar, H., and Nossal, R. J., *Gen. Physiol.*, **55**, 119-133 (1970).

⁴ *Membranes, 2, Lipid Bilayers and Antibiotics* (edit. by Eisenman, G.) (Marcel Dekker, New York, 1973).

cell types in the anterior lobe but leading in particular to proliferation of prolactin cells¹. Bromocryptine inhibits release of prolactin^{2,3} and also inhibits DNA synthesis in the oestrogen-stimulated pituitary gland⁴. In studying the pituitary response to oestrogen during a prolonged period of bromocryptine inhibition, we have observed a close relationship between effects on serum prolactin levels and on pituitary mitotic activity. Our findings lend support to the idea that secretory behaviour influences mitotic activity in prolactin cells⁴.

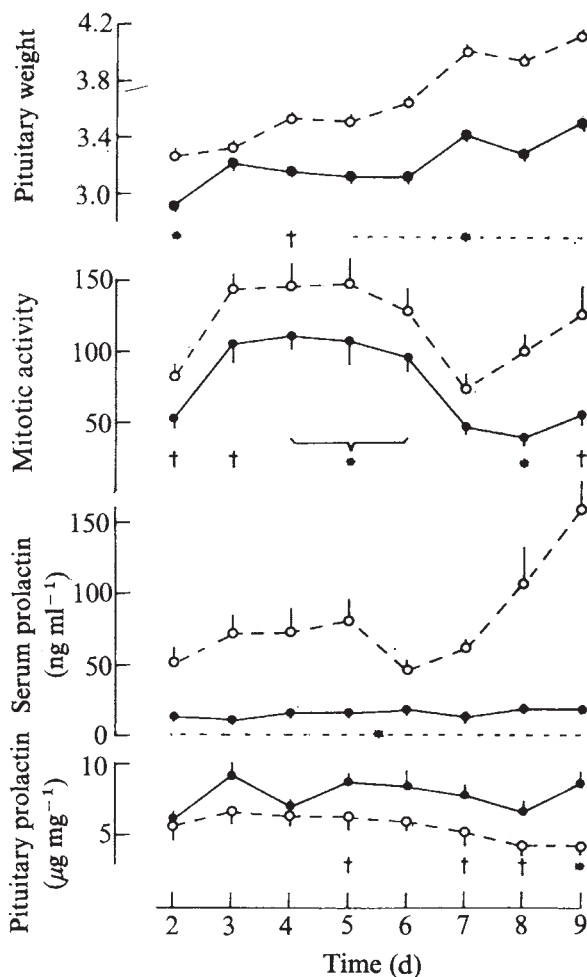


Fig. 1 Effects on male rats of a single dose of DESD (12 mg subcutaneously given on day 0) with (●) and without (○) bromocryptine (0.3 mg per 100 g body weight subcutaneously, given daily from day -1 to +8). Mean values ($\pm$ s.e.) in groups of seven rats for pituitary weight (mg per 100 g body weight) mitotic activity (mitotic figures mm⁻² pituitary section $\times$ pituitary weight) serum prolactin and pituitary prolactin concentration. Significance of difference (Student's t test): *, $P < 0.01$; †, $P < 0.05$.

Male Sprague-Dawley rats (3-4 months old), maintained in a light- and temperature-controlled room on tap water and a pellet diet *ad libitum*, were given a single subcutaneous dose of 12 mg diethylstilboestrol dipropionate (DESD) in oil on day 0. On day -1, and daily thereafter, the rats were given either bromocryptine (0.3 mg per 100 g body weight) or vehicle subcutaneously at 1600. On each of days 2-9, a test group (given DESD and bromocryptine) and a control group (given DESD and vehicle), seven rats each, were given colchicine in water (0.1 mg per 100 g body weight) intraperitoneally at 0900. Rats were anaesthetised with chloroform 1-1.5 h later and bled by section of the neck arteries until dead. Blood was kept for radioimmunoassay of prolactin and growth hormone. Pituitary glands were weighed and halved sagittally; one half was used for radioimmunoassay for prolactin and growth hormone, the other was fixed in Bouin-Hollande and 5 μ m sections cut and stained with haematoxylin and eosin. Two to

Effects of oestrogen and bromocryptine on *in vivo* secretion and mitosis in prolactin cells

THE response of the rat pituitary gland to oestrogen includes increased mitotic and secretory activity affecting several of the

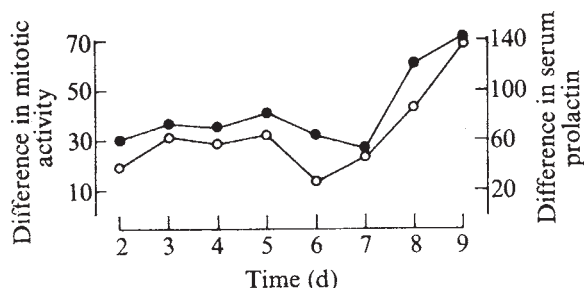


Fig. 2 Differences on each day in mean pituitary mitotic activity (●) and in mean serum prolactin (○) between rats given DESD and bromocryptine, and rats given DESD and vehicle.

three sections of each hemi-pituitary gland (50,000–70,000 cells) were examined for cells in mitosis.

The pituitary response to the single dose of DESD (Fig. 1) was similar to that described previously⁴—increasing pituitary weight and serum prolactin concentrations, and a later rise in serum growth hormone. The first 'wave' of mitotic activity subsided partially by day 7 and the subsequent rise was accompanied by rising serum prolactin and growth hormone. Bromocryptine inhibited the oestrogen-induced gain in pituitary weight, markedly reduced serum prolactin levels (Fig. 1) and had little effect on serum growth hormone. Pituitary prolactin concentrations were raised by bromocryptine whereas growth hormone showed no consistent change. Pituitary mitotic counts were reduced by bromocryptine on each of days 2–9, although the differences between test and control groups varied considerably. These differences were compared with those in serum prolactin and, for this purpose, the mitotic activity in each pituitary gland was derived by multiplying mitotic index (number of cells in mitosis per mm² pituitary section) by pituitary weight. The differences on each day in mean mitotic activity and in mean serum prolactin followed a similar pattern (Fig. 2) and were significantly correlated (Fig. 3).

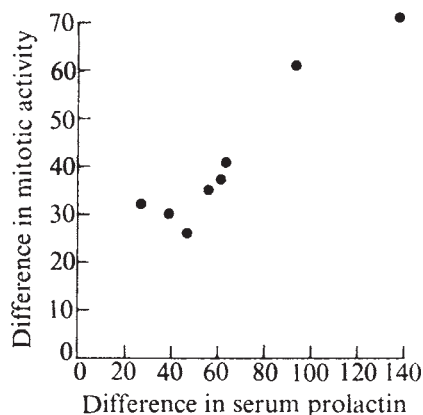


Fig. 3 Plot of differences induced by bromocryptine in mean pituitary mitotic activity against difference in mean serum prolactin on each of days 2–9. $r = +0.94$; $P < 0.001$.

Our findings suggest that bromocryptine inhibited proliferation of prolactin cells, thus accounting for the smaller gain in pituitary weight and the lower mitotic activity when compared with the effects of oestrogen alone. Functionally, the prolactin cells under the influence of bromocryptine and oestrogen maintained low serum prolactin levels and high intracellular concentrations. The oestrogenised pituitary glands, without bromocryptine, stored less and released more prolactin. The close correlation between the inhibitory effects of bromocryptine on mitotic activity and on serum prolactin may be interpreted in various ways. A direct effect of bromocryptine on DNA synthesis is a possibility but, to account for our findings, such an effect would have to be restricted to

the prolactin cells, enabling them to continue to maintain intracellular stores of prolactin and low but measurable serum prolactin levels. In inhibiting release of prolactin, bromocryptine could be acting by lowering pituitary cyclic AMP levels, this substance having been shown to stimulate prolactin release^{5,6}. Altered cyclic AMP levels may then influence DNA synthesis but, from studies on other tissues^{7,8}, a rise rather than a fall in cyclic AMP would be expected to lower pituitary DNA synthesis. Hypothalamic factors influenced by bromocryptine and oestrogen could also be involved in control mechanisms acting on mitotic⁹ as well as secretory activity¹⁰ in the pituitary gland. The two agents could be specific antagonists at either pituitary or hypothalamic levels. As the pituitary glands in the rats given bromocryptine maintained the higher prolactin concentrations, however, the effect of oestrogen on prolactin production did not seem to be inhibited by bromocryptine. There remains the possibility that bromocryptine acts exclusively by inhibiting prolactin release and that this in turn leads to inhibition of mitosis.

We reported previously⁴ that bromocryptine inhibition of DNA synthesis in oestrogenised pituitary glands followed the effects on prolactin release and suggested that the bromocryptine-induced rise in intracellular prolactin concentration could be a factor leading to inhibition of DNA synthesis. Our findings may mean that prolactin cells cannot divide under the influence of oestrogen unless they are free to release prolactin. An intracellular negative feedback may exist therefore between intracellular concentrations of the hormone produced and DNA synthesis. Such a mechanism would serve to inhibit cell division in cells containing adequate stores of their product and to trigger DNA synthesis when stores were depleted as a result of release exceeding production.

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- Gersten, B. E., and Baker, B. L., *Am. J. Anat.*, **128**, 1–20 (1970).
- Pasteels, J. L., Dangay, A., Frerotte, M., and Ectors, F., *Annls Endocr.*, **32**, 188–192 (1971).
- Shaar, C. J., and Clemens, J. A., *Endocrinology*, **90**, 285–288 (1972).
- Davies, C., Jacobi, J., Lloyd, H. M., and Meares, J. D., *J. Endocr.*, **61**, 411–417 (1974).
- Lemay, A., and Labrie, F., *FEBS Lett.*, **20**, 7–10 (1972).
- Nagasawa, H., and Yanai, R., *J. Endocr.*, **55**, 215–216 (1972).
- Masui, H., and Garren, L. D., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 3206–3210 (1971).
- Burger, M. M., Bombik, B. M., Breckenridge, B. McL., and Sheppard, J. R., *Nature*, **239**, 161–163 (1972).
- Hymer, W. C., Mastro, A., and Griswold, E., *Science*, **167**, 1629–1631 (1970).
- Meites, J., and Clemens, C. W., *Vitam. Horm.*, **30**, 165–221 (1972).

Metronidazole (Flagyl): iron catalysed reaction with sulphhydryl groups and tumour radiosensitisation

THE possibility of using hypoxic cell radiosensitising drugs as adjuncts in the radiotherapy of some forms of cancer has received renewed attention^{1–4}. In particular administration of metronidazole (Flagyl, May and Baker Ltd) to tumour bearing mice before exposure to X rays, has been found to reduce

the radiation dose required to control 50% of the tumours by a factor of 1.2–1.3 (refs 5 and 6). Preliminary clinical trials are now in progress⁷. The drug was originally chosen for study because of its constituent nitro group (which suggested it was a strong radical oxidant) and its extremely favourable pharmacological and toxicological properties⁸. Previously the activity of a large number of radiosensitisers had been attributed to their ability to react with intracellular sulphhydryl compounds^{9,10}. The ability of the cell to repair radiation-induced free radical lesions by hydrogen¹¹ or electron donation¹² would thus be lowered. Measurements¹³ of anoxic solutions over a period of 2 h did not, however, show any appreciable reaction between metronidazole (8×10^{-4} M) and the sulphhydryl groups of cysteamine (8×10^{-4} M) or dithiothreitol (4×10^{-4} M). Recent associated pharmacological studies with rat intestinal contents have prompted us to re-examine the reaction of metronidazole with sulphhydryl compounds and to investigate the possibility of catalysis by ferrous ions. We now report experiments which show that the drug reacts with an iron–cysteine complex over time scales of the order of a second. A drug–iron–cysteine complex is formed which is subsequently reduced over a period of minutes.

Solutions containing cysteine hydrochloride (0.01–0.1 M, titrated to $\text{pH} = 7.5 \pm 0.1$ by addition of KOH), and metronidazole (10^{-3} M) were purged with nitrogen in the absence and presence of ferrous sulphate (0.2–1 mM). Aliquots (0.5 ml) were removed at intervals and the concentration of metronidazole determined spectrophotometrically after extraction into ethyl acetate (5 ml). Unlike its reduction products the drug has an absorption maximum at 320 nm ($\text{max } \epsilon = 9,310 \text{ M}^{-1} \text{ cm}^{-1}$) characteristic of its nitro substituent (Fig. 1a). In the absence of ferrous ions, negligible removal of metronidazole occurred over a period of 2 h. In solutions containing the metal ion, however, levels of metronidazole decreased exponentially with

Fig. 1 A, Absorption spectra of ethyl acetate extracts taken at various times from nitrogen purged solutions originally containing cysteine (75 mM) ferrous sulphate (500 μM) and metronidazole (1 mM) $\text{pH} = 7.5$. a, After 5 min; b, 20 min; c, 40 min. B, Absorbance of extracts from solutions originally containing 0.1 M cysteine, metronidazole (1 mM) and ferrous sulphate. a, 0; b, 600 μM ; c, 1 mM. C, Plot of reciprocal half lives (min^{-1}) for metronidazole removal against ferrous sulphate concentration ($\times 10^{-4}$ M), 0.1 M cysteine. D, Plot of reciprocal half lives (min^{-1}) for metronidazole removal against cysteine concentration ($\times 10^{-2}$ M), 500 μM ferrous sulphate.

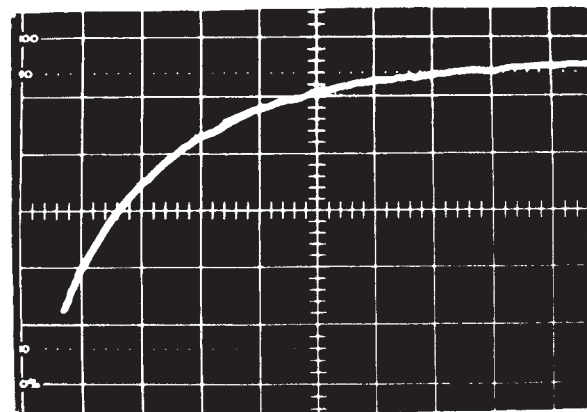
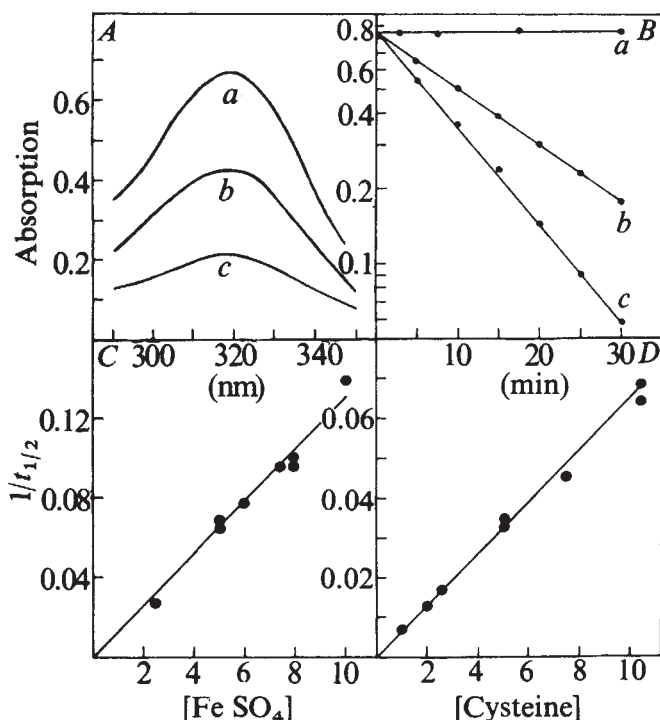
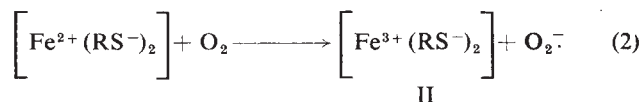
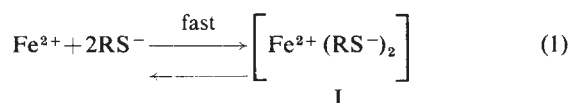
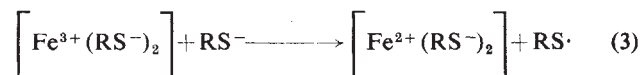


Fig. 2 Oscillogram obtained at 500 nm on mixing solutions containing metronidazole, cysteine and ferrous ion. (Final concentrations 25 mM, 0.1 M and 2 mM respectively, see text). Ordinate: transmission, 2.2% per large division; abscissa: time 1 s per large division.

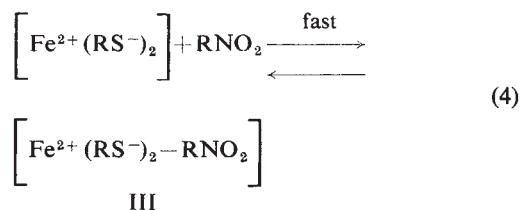
half lives ($t_{1/2}$) ranging from 6 to 140 min, and varying inversely with both ferrous ion and cysteine (RS^-) concentration (Fig. 1). Following preparation the solutions rapidly developed a purple colour, which later decayed during the experiment. Information concerning the rate of formation of the colour was subsequently obtained using a stopped-flow apparatus (Nortech Ltd). On mixing equal volumes of nitrogen purged solutions, one containing metronidazole (RNO_2 , 2×10^{-2} or 5×10^{-2} M), the other cysteine hydrochloride (0.1 M) and ferrous sulphate (4×10^{-3} M, $\text{pH} = 7.5 \pm 0.1$) the light transmitted at 500 nm decreased exponentially (Fig. 2). The decay half life varied inversely with metronidazole concentration: $\text{RNO}_2 = 10^{-2}$ M and 2.5×10^{-2} M, $t_{1/2} = 1.3$ and 2.3 s respectively. The above experiments were also undertaken with oxygen saturated solutions. Negligible removal of metronidazole occurred although the solutions again became coloured. A similar colour has been previously observed in oxygenated solutions in the absence of metronidazole and can be attributed^{14,15} to a cysteine–iron complex (II) formed according to:



and decaying by the chain propagating step:

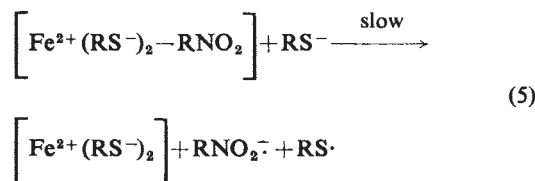


The kinetics of metronidazole reduction in the absence of oxygen are compatible within an analogous scheme but with the rapid formation of an intermediate coloured drug–iron–cysteine complex (III) following reaction (1).



Since for reaction (1) the equilibrium constant $K^e > 10^8$ the

rate-determining step for metronidazole reduction can be attributed to the reaction:

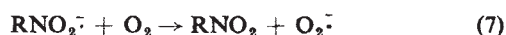


provided for reaction (4) the equilibrium constant is small, that is $K_c < \text{or} \ll 0.1$.

Although the present studies provide no information concerning the fate of the radical products from reaction (5) it is probable that cysteine is formed according to:



The subsequent reactions of RNO_2^- however are less clear. It has been suggested that in the absence of oxygen the radical can react with nucleophilic centres and this could lead to cell death¹⁶. This would provide a unifying mechanism for its extremely selective cytotoxic action on anaerobic organisms (aerobes are damaged only at higher concentrations) and its radiosensitising effect on hypoxic but not on normally oxygenated tissue. Pulse radiolysis studies¹⁶ have shown that the one electron redox potential of the oxygen-superoxide couple $E^\circ (\text{O}_2/\text{O}_2^-)$ is greater than that of the metronidazole and metronidazole radical-anion couple $E^\circ (\text{RNO}_2/\text{RNO}_2^-)$. Thus in the presence of oxygen the reaction:



can occur leading to removal of the damaging RNO_2^- and the reformation of original drug. In the present studies this is in agreement with the lack of removal of metronidazole in oxygen purged solutions. In oxygen free solutions it is possible, also, that the reaction:



takes place leading to further cysteine destruction, although the possible inter-radical reactions:



cannot be ruled out.

Whatever the fate of these radicals, however, it is clear that the reactions of metronidazole with cysteine and probably other sulphhydryl compounds in the presence of ferrous ions are sufficiently fast to warrant consideration in the interpretation of the drug's mechanism of radiosensitising and chemotherapeutic action.

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- ¹ Adams, G. E., *Br. Med. Bull.*, **29**, 48–53 (1973).
- ² Adams, G. E., Asquith, J. C., Watts, M. E., and Smithen, C. E., *Nature*, **239**, 23–24 (1972).
- ³ Denekamp, J., and Michael, B. D., *Nature*, **239**, 21–23 (1972).
- ⁴ Reuvers, A. P., Chapman, J. D., and Borsa, J., *Nature*, **237**, 402–403 (1972).
- ⁵ Begg, A. C., Sheldon, P. W., and Foster, J. L., *Br. J. Radiol.*, **47**, 399–404 (1974).
- ⁶ Stone, H. B., and Withers, H. R., *Radiology*, **113**, 441–444 (1974).
- ⁷ Uratson, R. C., Sturminwind, J., Robin, H., Band, P. R., Chapman, J. D., *Br. J. Radiol.*, **47**, 297–299 (1974).
- ⁸ Foster, J. L., and Willson, R. L., *Br. J. Radiol.*, **46**, 234–235 (1973).
- ⁹ Bridges, B. A., *Nature*, **188**, 415 (1960).

- ¹⁰ Ashwood-Smith, M. J., Robinson, D. M., Barnes, J. H., and Bridges, B. A., *Nature*, **216**, 137–139 (1967).
- ¹¹ Alexander, P., and Charlesby, A., *Radiobiology Symp.*, 49–58 (Butterworths, London, 1954).
- ¹² Willson, R. L., Wardman, P., and Asmus, K. D., *Nature*, **252**, 323–324 (1974).
- ¹³ Asquith, J. C., Foster, J. L., Willson, R. L., Ings, R. J. M., and MacFadzean, J. A., *Br. J. Radiol.*, **47**, 474–481 (1974).
- ¹⁴ Lanfron, H., and Neilson, S. O., *J. Am. Chem. Soc.*, **79**, 1966–1970 (1957).
- ¹⁵ Schubert, M., *Biochem. J.*, **40**, 4077–4085 (1932).
- ¹⁶ Willson, R. L., Cramp, W. C., and Ings, R. M. J., *Int. J. radiat. Biol.*, **26**, 557–569 (1974).

Survival of orally administered *E. coli* K12 in alimentary tract of man

VIEWS have recently been expressed that some aspects of genetic engineering research should not be allowed to continue because of their possible dangers to human health^{1,2}. For example, objection has been taken to the laboratory practice of incorporating genetic regions of foreign DNA in bacterial plasmids and introducing them into organisms whose normal habitat is the alimentary tract, because such organisms may accidentally gain access to the alimentary tract of man, colonise it and/or transmit the plasmids during conjugation to the resident intestinal flora. Consequently, we have investigated this matter in regard to *Escherichia coli* K12, the intestinal organism most frequently used as the recipient of plasmids into which foreign DNA is incorporated, using a human volunteer in whose alimentary

Table 1 Concentration of different strains of *E. coli* K12 in faeces following oral administration

K12 strain	No. of viable organisms ($\log_{10}$ per g faeces voided on the following days after administration)				
	1	2	3	4	5
Experiment 1					
711amp ^r	7.3	5.5	4.5	3.0	0
JRW nal ^r	6.2	5.0	4.0	2.0	0
328Chl ^r	6.2	4.5	3.0	2.0	0
Proto rif ^r	6.4	4.7	3.8	0	0
C600str ^r	6.2	4.5	3.0	0	0
J5 Neo ^r	5.6	5.3	4.3	0	0
410spc ^r	5.0	0	0	0	0
Resident <i>E. coli</i>	7.5	7.2	8.0	6.9	6.4
Experiment 2					
200rif ^r	6.6	5.0	3.5	2.0	0
C600nal ^r	6.5	5.1	0	0	0
Proto amp ^r	6.0	4.0	4.2	2.2	0
410spc ^r	6.0	3.3	0	0	0
711str ^r	4.8	4.2	3.2	0	0
Resident <i>E. coli</i>	7.5	7.0	ND	ND	ND
Experiment 3					
711nal ^r Tet ^r F ⁺ ,I ⁺ and A2 ⁺ *	7.3	5.3	3.7	1.3	0
711amp ^r	7.2	5.7	3.7	1.9	0
Proto amp ^r	5.2	<4.0	<2.0	0	0
H123 rif ^r †	8.2	7.8	6.5	3.4	2.4
Resident <i>E. coli</i>	5.7	6.5	6.9	6.4	6.4

Each strain was administered in doses of approximately $\log_{10}$ 9.0 viable organisms of an aerated broth culture that had been incubated at 37 °C for 24 h. $0 = <\log_{10}$ 1.0. Viable *E. coli* counts were performed on the faecal specimens within 0.5 h of being voided, by the method of Miles and Misra³, modified to permit the estimation of low concentrations of organisms. Ordinary MacConkey's agar was used for enumerating the total *E. coli*, and this medium containing antibiotics (20 $\mu\text{g ml}^{-1}$) for enumerating the different kinds of antibiotic-resistant *E. coli*. When the majority of the *E. coli* organisms in a specimen was not expected to be the resident strain, which was sulphonamide resistant, the latter was counted on Diagnostic Sensitivity Test Agar (Oxoid) containing 50 $\mu\text{g ml}^{-1}$ chlorsulphapyridazine. In experiment 3, Tet transfer from the Tet^r 711 strains to the resident strain was sought by inoculating the faecal specimens heavily on to plates of MacConkey's agar containing tetracycline and applying sulphonamide disks to their surfaces; MacConkey's agar containing tetracycline and rifampicin was used for detecting transfer to strain H123.

*This was a mixture of three strains possessing three different transfer factors, F, I or A2.

†This was a strain of *E. coli*, not K12, recently isolated from the faeces of a healthy human being.

ND, Not determined.

Table 2 Concentration of *E. coli* K12 711*nal*^r organisms in the faeces following oral administration at different dose levels

Approximate no. of viable organisms administered (log ₁₀)	No. of viable organisms (log ₁₀ per g faeces voided on the following days after administration)											
	1			2			3			4		
9.0	7.7	6.5	5.3	6.5	4.5	5.0	0	1.0	0	0	0	0
	3.5	3.2	—	2.8	3.2	—	2.0	2.7	—	0	0	—
8.0	4.0	3.9	3.5	3.3	2.7	2.5	1.2	0	1.3	0	0	0
7.0	2.7	1.3	0	0	1.0	0	0	0	0	0	0	0
6.0	0	0	0	0	0	0	0	0	0	0	0	0
9.5*	7.3	5.2	5.2	5.5	4.5	4.5	3.7	2.8	1.3	1.3	0	0
	5.0	4.3	4.3	4.2	3.3	4.2	0	2.0	0	0	0	0

Each dose level was tested on three to six occasions; the results for each test at each dose level occupy the same relative position in each column, for example those found on days 1, 2, 3 and 4 for the first dose level of 9.0 tested were 7.7, 6.5, 0 and 0, respectively.

*Equal mixture of organisms of *E. coli* K12 711Tet^r possessing transfer factors F, I and A2.

For other details see Table 1.

tract transfer of plasmids coding for antibiotic resistance (R factors) had previously been demonstrated following the consumption of wild strains of antibiotic-resistant *E. coli*³. Even when much higher concentrations of *E. coli* K12 organisms than would be expected to be accidentally consumed in a laboratory were taken, they did not colonise the alimentary tract but organisms of some strains survived better than others; no evidence of transfer of plasmids from these K12 organisms to the resident *E. coli* of the alimentary tract was found.

Seven mutants of K12 requiring different nutrients for growth, and a prototrophic form of this strain, Proto, were studied. To determine the number of organisms of several of these strains in the same specimen of faeces, mutants resistant to different antibiotics were prepared from them. These included nalidixic acid (*nal*^r), streptomycin (*str*^r), spectinomycin (*spc*^r), ampicillin (*amp*^r) and rifampicin (*rif*^r). Two of the K12 strains, 328 and J5, were differentiated by their possession of plasmids determining resistance to chloramphenicol (Chl^r) or to neomycin (Neo^r). Some of the K12 strains were also differentiated by the inability of some of them to ferment lactose (*lac*⁻). Three of the strains were also made thymine-dependent (*thy*⁻) because *thy*⁻ salmonella organisms had been found to be poorer colonisers of the alimentary tract than *thy*⁺ ones (H.W.S. and J. S. Tucker, unpublished). A plasmid determining tetracycline-resistance (Tet) was implanted in a *nal*^r mutant of one of the *lac*⁻ K12 strains, 711, using one or other of three transfer

factors, F (refs 4 and 5), I (Ildr 16(I), (ref. 6) and A2 (ref. 7); the resulting strains, 711 *nal*^r Tet⁺F⁺, 711 *nal*^rTet⁺I⁺ and 711 *nal*^rTet⁺A2⁺ were used for studying the possibility of plasmids being transferred from ingested *E. coli* K12 organisms to the *E. coli* resident in the alimentary tract of the volunteer. In *in vitro* mating studies, using the method of Smith⁸, all three strains could transmit Tet to the majority of *E. coli* K12 recipient organisms; in the Tet⁺F⁺ strain, Tet and F were linked. The resident *E. coli* during the whole study were dominated by one strain that possessed a transmissible plasmid determining sulphonamide resistance; it was usually present in concentrations of 10⁶–10⁸ viable organisms per g faeces.

The results of three experiments in which groups of antibiotic-resistant mutants of the eight different *E. coli* K12 strains were consumed simultaneously, each in doses of approximately log₁₀ 9.0 viable organisms, and their concentrations in the faeces subsequently estimated are summarised in Table 1. None of the strains persisted in the faeces for longer than 4 d; the highest concentrations were found on the first day after consumption, progressively decreasing concentrations being found on subsequent days until the organisms were no longer isolated; some strains, notably 410 *spc*^r, persisted more poorly than others. By contrast, the high concentrations of organisms of the resident *E. coli* strain remained relatively constant throughout the three experiments. A human faecal *E. coli* strain, H123, was included in the third experiment. At each daily examination it was present in much higher concentrations than any of the K12 strains and it persisted in the faeces for 7 d. In this experiment there was no evidence of transfer of the tetracycline plasmid to strain H123 or to the resident *E. coli* strain from the three Tet⁺711*nal*^r strains, 711Tet⁺F⁺, 711Tet⁺I⁺, and 711Tet⁺A2⁺. In *in vitro* studies these three strains transferred their tetracycline resistance plasmids to H123 and the resident *E. coli* strain at approximately the same rate, about one in 500 organisms of these strains receiving the plasmid.

The results of consuming on three to six occasions at each of four different dose levels 711*nal*^r (one of the K12 strains that survived best in the alimentary tract, Table 1), are summarised in Table 2. In general, the larger the dose consumed the greater were the concentrations of organisms found in the faeces. Considerable variations in faecal concentrations were, however, sometimes found when the same number of organisms were consumed on different occasions. For example, the highest concentration of 711*nal*^r organisms found in the faeces following the consumption of log₁₀ 9.0 organisms was log₁₀ 7.7. When the same dose was consumed on another occasion the highest concentration found was log₁₀ 3.2. Very few organisms

Table 3 Concentration of organisms of three *thy*⁻ and one *thy*⁺ K12 strain in faeces following oral administration at different dose levels

Approximate no. of viable organisms administered (log ₁₀)	Strain	No. of viable organisms (log ₁₀ per g faeces, voided on the following days after administration)											
		1			2			3			4		
10.0	410 <i>spc</i> ^r <i>thy</i> ⁻	4.8			0			0			0		
	Proto <i>amp</i> ^r <i>thy</i> ⁻	6.2			3.5			3.0			0		
	711 <i>str</i> ^r <i>thy</i> ⁻	6.5			5.0			3.4			0		
	711 <i>nal</i> ^r <i>thy</i> ⁺	7.8			6.4			4.4			2.7		
	Resident <i>E. coli</i>	7.5			6.9			6.9			6.7		
9.0	410 <i>spc</i> ^r <i>thy</i> ⁻	2.7	3.2	0	0	0	0	0	0	0	0	0	0
	Proto <i>amp</i> ^r <i>thy</i> ⁻	2.7	2.8	2.2	1.3	2.4	0	0	0	0	0	0	0
	711 <i>str</i> ^r <i>thy</i> ⁻	4.3	4.0	3.2	3.2	2.8	2.3	0	0	0	0	0	0
	711 <i>nal</i> ^r <i>thy</i> ⁺	5.2	6.2	4.0	4.4	5.7	3.2	1.6	4.2	2.6	0	3.4	1.8
	Resident <i>E. coli</i>	7.0	7.2	6.7	6.2	7.0	5.7	6.4	7.2	6.7	6.7	6.5	6.3
8.0	410 <i>spc</i> ^r <i>thy</i> ⁻	1.6	0	0	0	0	0	0	0	0	0	ND	0
	Proto <i>amp</i> ^r <i>thy</i> ⁻	3.5	2.0	2.0	1.9	0	0	0	0	0	0	ND	0
	711 <i>str</i> ^r <i>thy</i> ⁻	3.3	2.3	2.0	2.3	1.3	0	0	0	0	0	ND	0
	711 <i>nal</i> ^r <i>thy</i> ⁺	5.2	4.4	3.5	4.5	3.7	2.0	2.5	3.0	1.7	1.6	ND	0
	Resident <i>E. coli</i>	7.3	7.7	7.7	7.5	7.4	7.3	7.8	7.7	7.0	7.5	ND	6.5

For other details see Tables 1 and 2.

All culture media used in these experiments were supplemented by the addition of 50 µg ml⁻¹ thymine.

were found in the faeces following the consumption of $\log_{10}$ 7.0 viable organisms of this strain and none was found when $\log_{10}$ 6.0 was consumed even though the cultural methods used enabled the isolation of organisms present in concentrations of 10 per g of faeces. In this experiment, mixtures containing $\log_{10}$ 9.0 viable organisms of each of the three 711nal⁺Tet⁺ strains possessing transfer factors F_I or A2 were consumed on three separate occasions; transfer of the tetracycline plasmid to the resident *E. coli* strain was never observed.

The ability of three thymine-dependent (*thy*⁻) K12 strains and the *thy*⁺711nal⁺ strain to survive in the alimentary tract of the human volunteer are compared in Table 3. At all dose levels tested, the *thy*⁻ strains, especially strain 410 *spc*⁺, survived more poorly than the *thy*⁺711nal⁺ strain.

The pattern of faecal excretion of the K12 organisms in all three experiments in which the highest concentrations of organisms were found on day 1 after ingestion, then progressively lower ones on subsequent days and none after day 4 suggested that in the main, the organisms were simply passing through the alimentary tract. The actual concentrations found in the faeces on some occasions indicated that the process was often accompanied by high mortality. The failure to demonstrate transfer of the tetracycline resistance plasmid from the 711nal⁺ organisms possessing the highly efficient transfer factors F_I and A2 to the resident *E. coli* flora was not unexpected because experiments in animals⁹ had shown that to obtain substantial transfer of R factor required the use of organisms that were not only efficient donors and recipients but also good colonisers of the alimentary tract. Previous work using more robust strains of *E. coli* than K12 (refs 3, 10–12) has also demonstrated the difficulty of transferring R factors experimentally in the human alimentary tract.

The present work is open to the criticism that it was done on only one human being. Quite different results might have been obtained in other human beings. Even so, it is probable that the relative ability of different K12 strains to survive in the alimentary tract would be the same in most human beings. Note that when several strains were tested on the same occasion some, especially 410*spc*⁺*thy*⁻, survived more poorly than others in the alimentary tract of the human volunteer. Everything else being equal, it would therefore seem prudent to use strains such as these in potentially dangerous genetic engineering experiments, unless ones that survive even more poorly can be found.

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- ¹ Science, 185, 332 (1974).
- ² Nature, 250, 175 (1974).
- ³ Smith, H. W., Lancet, i, 1174 (1969).
- ⁴ Lederberg, J., Cavalli, L. L., and Lederberg, E. M., Genetics, 37, 720 (1952).
- ⁵ Hayes, W., J. gen. Microbiol., 8, 72 (1953).
- ⁶ Hardy, K. G., Meynell, G. G., Dowman, J. E., and Spratt, B. G., Molec. gen. Genet., 125, 217 (1973).
- ⁷ Smith, H. W., and Heller, E. D., J. gen. Microbiol., 78, 89 (1973).
- ⁸ Miles, A. A., and Misra, S. S., J. Hyg., Camb., 38, 732 (1938).
- ⁹ Smith, H. W., J. med. Microbiol., 3, 165 (1970).
- ¹⁰ Wiedemann, B., Knothe, H., and Doll, E., Zentbl. Bakt., I. Orig., 213, 183 (1970).
- ¹¹ Anderson, J. D., Gillespie, W. A., and Richmond, M. H., J. med. Microbiol., 6, 461 (1973).
- ¹² Ingram, L. C., Anderson, J. D., Arrand, J. E., and Richmond, M. H., J. med. Microbiol., 7, 251 (1974).

for plasmid transfer to the resident *E. coli*, in the human intestine. A preliminary summary of our observations was presented to the Working Party headed by Lord Ashby¹.

The K12 strain of *E. coli* was isolated from the faeces of a convalescent diphtheria patient in 1922, probably at Stanford Hospital, California. It was incorporated in the stock collection at Stanford University in 1925, and was given the catalogue designation K12. The question of its capacity for survival in the human intestine has become important since the call for an embargo on work with hybrid plasmids^{2,3}, because the organisms into which these plasmids are usually introduced is K12. If it can survive in the human intestine long enough to transfer a hybrid plasmid to the resident enterobacteria, DNA of non-bacterial origin (xeno-DNA) in such plasmids might be transferred to intestinal or other human cells. If the xeno-DNA were derived from a pathogenic microorganism such as a virus, which could cause human epidemic infections or malignancy (the properties of communicability and oncogenicity might be combined), work on such plasmids might present unacceptable biohazards in the absence of reliable safeguards.

We have carried out preliminary experiments to examine the efficiency of plasmid transfer to wild *E. coli* and salmonellae of human origin. *In vitro* transfer experiments were carried out using the donor strain *E. coli* K12 F⁻*lac*⁻, carrying the hybrid F' plasmid F⁻*lac*-T (where T is tetracycline resistance). This is a recombinant of F⁻*lac* with the T-Δ factor, and was produced intracellularly⁴. The T-Δ R factor was isolated from type 29 of *Salmonella typhimurium*⁵. As far as can be determined it has no molecular similarity with F⁻*lac*, and the two plasmids belong to different compatibility groups, F_I in the case of F⁻*lac*, and I₁ in that of T-Δ. F⁻*lac*-T behaves as an ordinary F' factor. It is

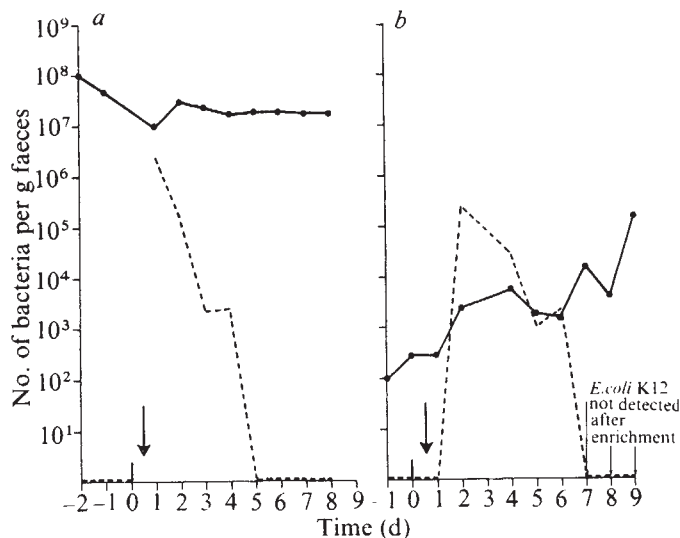


Fig. 1 Survival of *E. coli* K12 in human subjects after ingestion of: a, 3×10^{10} (subject 1); and b, 4×10^8 *E. coli* K12 F⁻*lac*⁻Nal^r (subject 2). ●, Resident coliforms; dotted line, *E. coli* K12. Subjects were healthy adults aged 27 to 60 yr, three males and five females. Faeces were tested for 2 to 3 d before the experiment to exclude the presence of nalidixic acid-resistant resident *E. coli*. None was found. Strain 14R519 in late exponential growth was administered (at arrow) in doses of about 10^8 to 10^{10} organisms in decimal steps. The dose was suspended in about half a pint of milk. Counts of 14R519 started 15 to 18 h later and continued until 3 to 4 d after the introduced strain was no longer detectable. Faecal specimens were emulsified 1 : 10 in saline. Serial dilutions were plated on plain MacConkey agar for total faecal *E. coli* counts. Counts were similarly carried out on MacConkey agar containing $40 \mu\text{g ml}^{-1}$ nalidixic acid to detect 14R519. In six subjects enrichment of 14R519 was attempted by incubation of faeces in broth containing nalidixic acid as soon as the strain was no longer detected by direct plating. It was demonstrated by this method in one subject only. All isolates of 14R519 were tested for sensitivity to phage $\phi 2$ of Cuzin⁶, which lyses K12 F⁻ strains but very few strains of wild *E. coli*. All *lac*⁻Nal^r isolates were sensitive to this phage. Strain 14R519 was not detected in the faeces of subject 8, who ingested 10^8 organisms.

Viability of, and transfer of a plasmid from, *E. coli* K12 in the human intestine

THE controversy over the ability of *Escherichia coli* K12 to survive in the human gut and to transfer plasmids to the indigenous flora, arose in relation to proposed research on bacterial plasmids and other genetic agents hybridised with DNA from organisms unrelated to the normal hosts of the plasmids or to the plasmids themselves. As the bacterial strain used in most studies of microbial genetics is *E. coli* K12, experiments were designed to investigate its viability, and its capacity

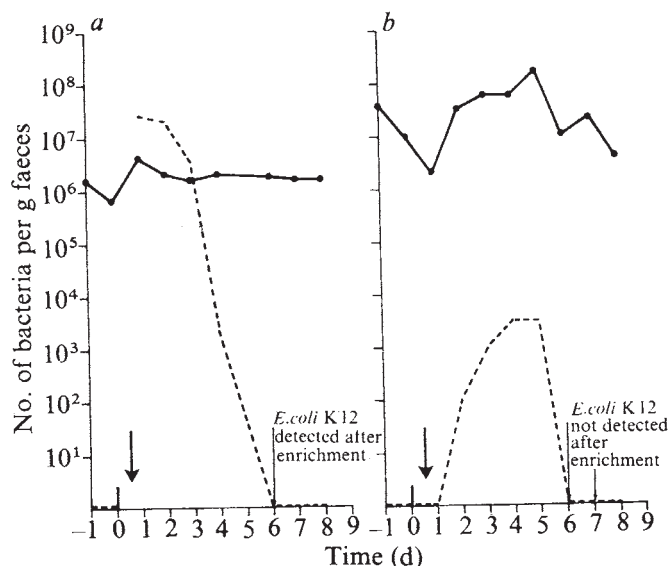


Fig. 2 Survival of *E. coli* K12 in human subjects after ingestion of: a, 3×10^8 (subject 3); and b, 9×10^6 *E. coli* K12 F-lac⁻Nal^r (subject 4). Key and methods as in Fig. 1.

derepressed and has the same transfer kinetics and compatibilities as the F-lac factor. It has been assumed that only the T region of T-Δ is present in F-lac-T, but this awaits detailed confirmation.

Recipient strains used in the *in vitro* experiments were: *E. coli* EC36 (a wild drug-sensitive strain of human origin, and a fair recipient of the F factor); *E. coli* EC1 (a human drug-sensitive strain and a poor recipient of F); *S. agama* (a relatively rare serotype in man—about 0.3% of total annual human salmonellosis in Britain—and a fair recipient of F); *S. typhimurium* phage type 36 (*S. typhimurium* is the commonest human and animal salmonella in most countries, type 36 being a rather poor recipient of F). There are very few good recipients of the F factor: K12 is one of these, and it is also an excellent recipient of F-lac-T. A K12 line which is lac⁻ and nalidixic acid-resistant (K12 lac⁻Nal^r), numbered 14R519, was therefore used as a control recipient in these experiments.

Table 1 shows the lowest numbers of K12 lac⁻ (F-lac-T) donor cells from which transfer of F-lac-T to each recipient strain was observed after 2, 6 and 20 h of mating. The donor dosage needed was less for the fair recipients EC36 and *S. agama* than for the poor recipients EC1 and *S. typhimurium* type 36,

Table 1 Quantitation of plasmid transfer to enterobacterial strains

Recipient strains	Minimum number of K12 lac ⁻ (F-lac-T) ml ⁻¹ to detect transfer to recipient in:		
	2 h	6 h	20 h
<i>E. coli</i> EC36	3×10^6	3×10^5	2×10^3
<i>E. coli</i> EC1	3×10^7	3×10^6	2×10^4
<i>S. agama</i>	7×10^5	2×10^5	< 13
<i>S. typhimurium</i> type 36	> 10^8	> 10^8	10^3
<i>E. coli</i> K12 lac ⁻ Nal ^r	< 5	< 5	< 5
14R519 (control)			

Bacto-dehydrated nutrient broth (Difco) was used in a concentration of 2% for routine incubation of bacterial cultures and mating mixtures. Oxoid MacConkey agar No. 3 was used for detecting transfer to the recipient strains. Selection for F-lac-T transfer to recipients was exercised with tetracycline, of which $10 \mu\text{g ml}^{-1}$ was incorporated in the MacConkey agar. Counter-selection against the K12 donor was effected by incubating the mating mixture with high titre phage T6, and spreading undiluted colicin E₂ on the plates. When the recipient was 14R519 the donor K12 strain was eliminated by incorporating $40 \mu\text{g ml}^{-1}$ nalidixic acid in the medium. About 10^1 to 10^8 organisms of the donor strain were added to a culture of each recipient in late exponential growth in nutrient broth.

but transfer of F-lac-T was poor throughout, except in *S. agama*, where a dose of < 13 donor cells was adequate after 20 h. There was little spread of F-lac-T through the recipient population. In contrast to the results obtained with the wild *E. coli* and salmonellae, transfer of F-lac-T to 14R519 was unrestricted from the smallest donor dose, which was five organisms. Free spread of F-lac-T in the 14R519 population was also observed, unlike the wild *E. coli* and salmonellae, where spread was minimal. We conclude therefore that transfer of the hybrid F-lac-T plasmid from K12 to wild enterobacterial strains is probably poor in general.

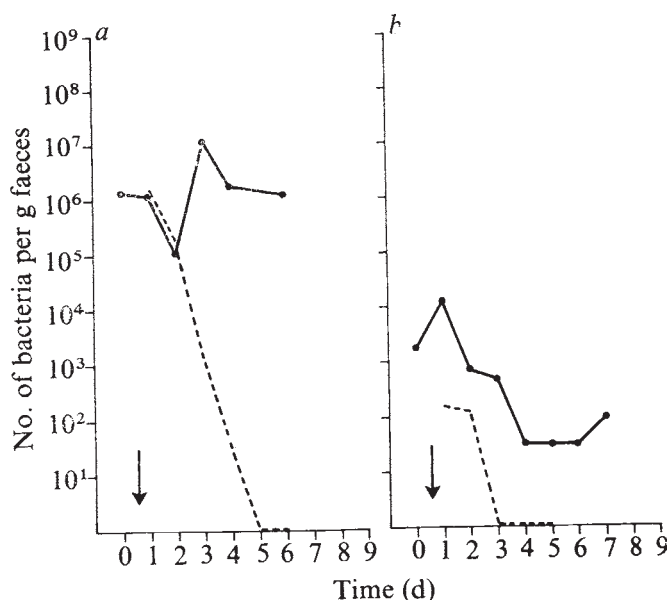


Fig. 3 Survival of *E. coli* K12 in human subjects after ingestion of: a, 3×10^8 (subject 5); and b, 3×10^6 *E. coli* K12 F-lac⁻Nal^r (subject 6). Key and methods as in Fig. 1.

To determine survival of K12 *in vivo*, *E. coli* K12 lac⁻Nal^r strain 14R519 was used. Figures 1–4 show that *E. coli* K12 can survive for some days when introduced into the human gastrointestinal tract. Starting with the highest dosage the minimum survival periods were as follows: 10^{10} , 4 d; 10^9 , 6 d; 10^8 , 6 d; 10^7 , 5 d; 10^6 , 4 d; 10^5 , 2 d; 10^4 , 3 d. The shortest survival duration was 2 d (dose 10^6) and the longest, 6 d (doses 10^8 and 10^9). Although the larger doses gave in general the longest survival times, this was not directly dose related, as the results with subjects 1 to 5 show.

In subjects 3 and 5 at least, multiplication of 14R519 probably occurred. If it is assumed that the organism is uniformly distributed throughout the faeces when the count is stable for a minimum of 24 h, and that the contents of the adult large intestine are of the order of 1 l, the total count of 14R519 in the large bowel during at least 24 h exceeded the dose initially ingested. The distribution of 14R519 could be monitored only at 15 to 24 h intervals, so that the multiplication can only be postulated. Nevertheless it is a reasonable extrapolation from our results.

To transfer a hybrid F' plasmid *in vivo*, the donor strain used was K12 lac⁻ (F-T), Enteric Reference Laboratory No. 35R169, the F-T plasmid being derived from the F-lac-T hybrid⁴ by loss or inactivation of the lac operon. The subject used for this experiment was a healthy adult male aged 29 yr, who had no tetracycline-resistant organisms in his faeces. Figure 5 shows that the resident *E. coli* count was 10^6 to 10^7 g⁻¹ and the donor strain 35R169 persisted for 4 d. Transfer of F-T occurred at a very low level: about $20 \text{ lac}^+ \text{F-T}^+$ organisms per g faeces were present 24 h after the donor strain was ingested. By the time the next specimen was tested 24 h later they had disappeared, and were not subsequently detected. Although we cannot absolutely exclude the possibility that *in vitro* transfer of F-T

had occurred when the faeces were emulsified in saline for plating, the precautions taken to prevent this—addition of phage T6, colicin E₂ and tetracycline to the saline—make it unlikely. We believe that the appearance of F-T in the resident *E. coli* was the result of *in vivo* plasmid transfer. Moreover, if tetracycline had been administered during the experiment, the transfer frequency of F-T to the resident flora would probably have been dramatically increased^{7,8}. We deliberately avoided this to preclude the possibility of biasing the result.

Unfortunately, it is only too clear that *in vivo* plasmid transfer is occurring constantly in man and animals. The prevalence of enterobacteria of all sorts, rich in R factors and other plasmids, bears witness to this, a state which is the result of the global pressure of antibiotics.

The results of our experiments are reasonably clear cut. Wild human *E. coli* and salmonellae are rather poor recipients of the F-lac-T hybrid. K12 can survive for a few days in the human intestine and may even multiply for a short while, but it is ultimately eliminated. The F-T hybrid can almost certainly be transferred to the resident *E. coli* in the human intestine. In the absence of antibiotic selection, however, *in vivo* transfer of F-T has a very low frequency, and F-T⁺ resident organisms do not persist, nor does the plasmid spread.

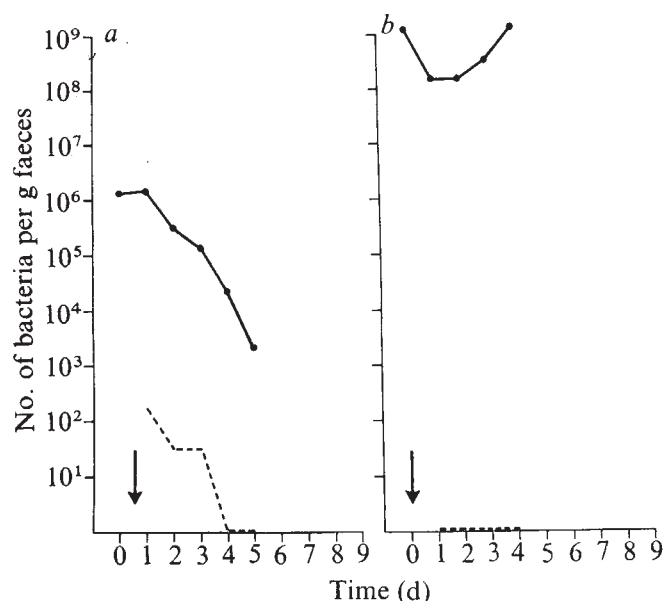


Fig. 4 Survival of *E. coli* K12 in human subjects after ingestion of: a, 3×10^4 (subject 7); and b, 3×10^3 *E. coli* K12 F-lac-Nal^r (subject 8). Key and methods as in Fig. 1.

The results show therefore that K12 can survive in the human intestine and can transfer hybrid plasmids to the resident *E. coli*¹. The fact that F-lac-T and F-T are purely bacterial hybrid plasmids probably does not prejudice the issue, since the transfer mechanism depends, not on the nature of the xenodna in a plasmid, but on the transfer factor itself, the F factor in the present instance. The low F-T transfer frequency *in vivo* even when the dose of K12 was 10^{10} organisms, indicates the remoteness of the possibility of such an event occurring by accident, which would probably involve the ingestion of only a few hundred or thousand bacteria. Obviously, more experiments along these lines need to be done, but our present findings suggest that in most instances experiments involving K12-carrying hybrid plasmids can safely be carried out with the exercise of the simple but effective techniques used in laboratories handling organisms such as the typhoid bacillus and similar pathogens. It is in any event an advantage for all who work with bacteria to be trained in such techniques, which not only protect against accidental infection but also add reliability to the experimental findings.

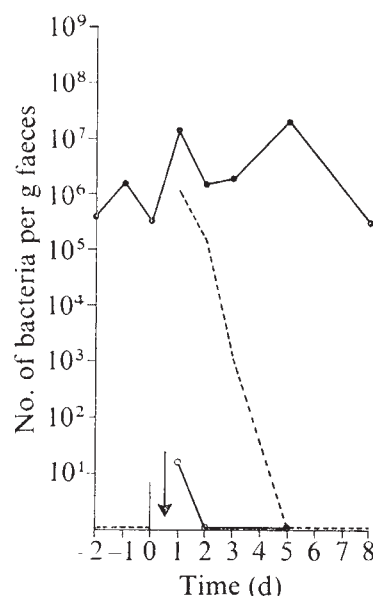


Fig. 5 Survival of K12 in a human subject (9) after ingestion of 10^{10} *E. coli* K12 lac-(F-T). ●, Resident coliforms; dotted line, *E. coli* K12; ○, resident coliforms carrying F-T. Strain 35R169 was administered in a dose of 10^{10} organisms (at arrow) in half a pint of milk in early evening. The faeces were examined the following morning, and daily thereafter. The organisms monitored were: the resident *E. coli*; 35R169; and the resident *E. coli* carrying the F-T plasmid. Faeces were emulsified in saline to a dilution of 1:10. Unchanged resident *E. coli* were counted by serial dilution on ordinary MacConkey agar plates. Strain 35R169 was counted by a similar technique on MacConkey agar containing $10 \mu\text{g ml}^{-1}$ tetracycline. Resident *E. coli* carrying the F-T plasmid were detected on MacConkey agar containing tetracycline. To minimise the possibility of the result being affected by *in vitro* transfer to the resident *E. coli* while the faeces were being suspended in saline, phage T6, colicin E₂ and tetracycline were incorporated in the saline used for suspension. Colicin E₂ was also spread on the MacConkey agar before mating mixtures were plated further, to ensure against survival of the donor strain. No donor colonies survived this selection. About 1 ml of the faecal suspension was plated, in 0.2 ml quantities on five plates, for the detection of F-T transfer.

If there is reason to suspect that the danger is of a higher order, the safety measures must be scaled up accordingly. Moreover, additional safeguards can be imposed on both K12 (or other host strains) and hybrid plasmids by the induction of mutations that would be lethal except in the desired experimental conditions.

I thank members of my staff who volunteered to be 'guinea pigs' in these experiments. This paper was presented at the International Conference on Recombinant DNA Molecules, Asilomar, Pacific Grove, California (February 24-27, 1975).

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- 1 Report, Cmdt 5880 (HMSO, London, 1974).
- 2 Berg, P. *et al.*, *Nature*, **250**, 175 (1974).
- 3 Berg, P. *et al.*, *Science*, **185**, 303 (1974).
- 4 Anderson, E. S., *Annls Microbiol. Inst. Pasteur, Paris*, **125A**, No. 3, 251 (1974).
- 5 Anderson, E. S., and Lewis, M. J., *Nature*, **208**, 843 (1965).
- 6 Cuzin, F., *C. r. hebdo Séanc Acad. Sci., Paris*, **260**, 6482 (1965).
- 7 Anderson, E. S., *Br. Med. J.*, **2**, 1289 (1965).
- 8 Anderson, J. D., Gillespie, W. A., and Richmond, M. H., *J. med. Microbiol.*, **6**, 461 (1973).

Plasmid RP4 as a vector replicon in genetic engineering

THERE is now considerable interest in the construction of plasmids containing DNA fragments from a variety of bacterial genera,

for purposes of studying the expression of the genetic potentialities of the incorporated bacterial DNA in different bacterial hosts. We report that the self-transmissible plasmid RP4 has only one site susceptible to cutting by the enzyme *EcoRI*, and should therefore be a very suitable bacterial DNA vector in such experiments.

The restriction endonuclease *EcoRI*, which cleaves double-stranded DNA to produce short, overlapping, self-complementary single-stranded ends^{1,2}, has been used to generate complementary single-stranded sequences between the plasmid vector and the DNA to be incorporated into the plasmid. The plasmid and bacterial DNA fragments are allowed to associate by hydrogen bonding, and the 3'-hydroxyl and 5'-phosphate ends are then covalently linked by the action of the enzyme DNA ligase³. Experiments described so far have been limited by the use of plasmids that are not self-transmissible as the DNA vector: the expression of 'foreign' DNA could be studied only in *Escherichia coli*, into which the 'recombinant' plasmid was introduced by transformation³⁻⁵.

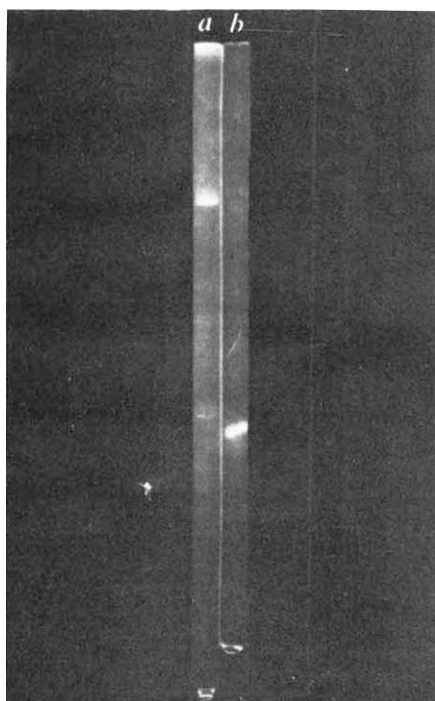


Fig. 1 Agarose gel electrophoresis of *EcoRI*-digested RP4 DNA. The covalently closed circular DNA tertiary form of plasmid RP4 was isolated using caesium chloride-ethidium bromide density gradient centrifugation^{11,14}. Part (0.5 µg) of this DNA was digested with *EcoRI* enzyme and prepared for electrophoresis³. A second DNA sample was similarly prepared, except that *EcoRI* treatment was omitted. Electrophoresis was carried out vertically, in a downward direction, in 1% agarose gels held in glass tubes, 150 mm × 5 mm internal diameter, for 18 h at 2 mA per tube and 20 °C (ref. 13). Gels were removed from the holders and photographed under ultraviolet illumination through a red filter. Exposure was 4 min at F4.5 on Polaroid type 107 film. *a*, Covalently closed circular RP4 DNA; *b*, RP4 DNA after *EcoRI* endonuclease treatment.

The potential usefulness of the technique would be enhanced by the use of a plasmid that is transmissible, by conjugation, between many bacterial genera. Such manipulations fall into the purview of the Ashby Committee Report⁶; unless the 'foreign' DNA carries genes relevant to pathogenesis, no special hazard is to be anticipated and the techniques of containment usual in Public Health Laboratories would be appropriate.

Plasmids of incompatibility group P have a uniquely wide host range; they transfer to, and replicate stably in, many bacterial genera, including *E. coli*, *Proteus* spp., *Pseudomonas* spp., *Neisseria* spp., the photosynthetic *Rhodospseudomonas*,

and the nitrogen-fixing *Azotobacter* and *Rhizobium* spp.⁷⁻¹⁰. Of the P group plasmids, RP4 has been most extensively studied^{7,11}. It has been shown that RP4 can incorporate 'foreign' bacterial and plasmid DNA which can be genetically expressed; Dixon *et al.*¹² introduced the nitrogen fixation genes, *nif*, from *Klebsiella pneumoniae* into RP4, and an RP4 derivative has been isolated into which trimethoprim and streptomycin resistance genes have been transposed from a plasmid of the I incompatibility complex (unpublished). These plasmids were constructed by *in vivo* genetic manipulations.

We now show that RP4 has only one site that is susceptible to cutting by the *EcoRI* endonuclease to produce linear monomeric DNA and, as it is probable that only one gene function

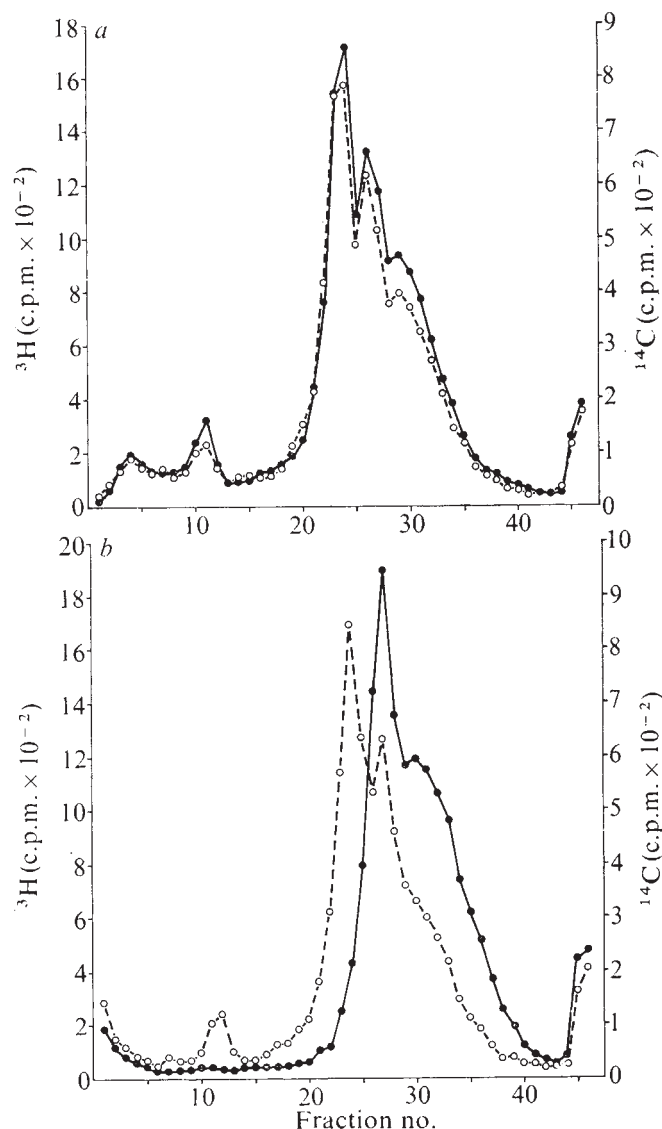


Fig. 2 Neutral sucrose gradient analysis of *EcoRI*-digested RP4 DNA. The covalently closed circular DNA tertiary form of plasmid RP4 was isolated as described in Fig. 1. This DNA spontaneously degraded to other tertiary forms on storage. For *EcoRI* digestion of DNA, 2 µl of endonuclease solution was added to 20 µl DNA solution (100 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, pH 7.5) and incubated at 37 °C for 30 min. Endonuclease activity was then destroyed by heating at 63 °C for 5 min¹⁵. DNA samples were analysed by sedimentation through a 4.6 ml 5 to 20% sucrose gradient¹⁴ at 100,000g and 20 °C for 120 min. Sedimentation was from right to left. Fractions (0.1 ml) were collected directly on to glass fibre disks and, after drying, washing and redrying, were assayed for radioactivity: ●, ³H; ○, ¹⁴C. *a*, ¹⁴C-labelled RP4 DNA plus ³H-labelled RP4 DNA. *b*, ¹⁴C-labelled RP4 DNA plus *EcoRI*-digested ³H-labelled RP4 DNA.

has been destroyed, this molecule should make an ideal vector for genetic engineering.

RP4 has a molecular weight of 36×10^6 (P. T. Barth, personal communication). Initial experiments using agarose gel electrophoresis^{5,13} showed that the covalently closed circular form of RP4 DNA was converted by digestion with *EcoRI* endonuclease to a single DNA band that migrated considerably faster than the untreated DNA (Fig. 1). We proved that *EcoRI* digestion converted RP4 DNA to the linear monomeric form, by comparing the rate of sedimentation of *EcoRI*-digested and untreated DNA through 5–20% neutral sucrose gradients. Figure 2a shows that untreated ¹⁴C-labelled RP4 DNA and ³H-labelled RP4 DNA cosediment; the bands that peak at fractions 11, 24 and 26 are the covalently closed, open circular and linear tertiary forms of RP4 DNA. The open circular and linear forms and the further degraded DNA (between fractions 28 and 38) were formed by spontaneous breakdown of the covalently closed circular DNA form on storage. After complete digestion by *EcoRI*, the ³H-labelled RP4 DNA cosedimented only with the linear form of ¹⁴C-labelled RP4 DNA (Fig. 2b). Using the equation $d_{oc}/d_{lin} = 1.0965 \times M^{0.01}$, where d_{oc} , d_{lin} are the distances sedimented from the meniscus by the open circular and linear tertiary forms of a plasmid of molecular weight M (P. T. Barth, personal communication); the molecular weight of the *EcoRI*-digested RP4 DNA was calculated to be 36×10^6 .

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- ¹ Hedgepeth, J., Goodman, H. M., and Boyer, H. W., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3448–3452 (1972).
- ² Mertz, J. E., and Davis, R. W., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3370–3374 (1972).
- ³ Cohen, S. N., Chang, A. C. Y., Boyer, H. W., and Helling, R. B., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3240–3244 (1973).
- ⁴ Chang, A. C. Y., and Cohen, S. N., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1030–1034 (1974).
- ⁵ Tanaka, T., and Weisblum, B., *J. Bact.*, **121**, 354–362 (1975).
- ⁶ Report of the working party on the experimental manipulation of the genetic composition of micro-organisms (HMSO, Cmd No. 5880, 1975).
- ⁷ Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. P., and Richmond, M. H., *J. Bact.*, **108**, 1244–1249 (1971).
- ⁸ Olsen, R. H., and Shipley, P., *J. Bact.*, **113**, 772–780 (1973).
- ⁹ Beringer, J., *J. gen. Microbiol.*, **84**, 188–198 (1974).
- ¹⁰ Hedges, R. W., Jacob, A. E., and Smith, J. T., *J. gen. Microbiol.*, **84**, 199–204 (1974).
- ¹¹ Hedges, R. W., and Jacob, A. E., *Molec. gen. Genet.*, **132**, 31–40 (1974).
- ¹² Dixon, R. A., Cannon, F. C., and Kondorosi, A., *Proc. soc. Gen. Microbiol.*, **2**, 43 (1975).
- ¹³ Sharp, P. A., Sugden, B., and Sambrook, J., *Biochemistry*, **12**, 3055–3063 (1973).
- ¹⁴ Jacob, A. E., and Hobbs, S. J., *J. Bact.*, **117**, 360–372 (1974).
- ¹⁵ Morrow, J. F., et al., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1743–1747 (1974).

Carcinogenicity and mutagenicity tests of some hair colourants and constituents

CONSIDERATION of exposure to drugs or other chemicals is part of the normal haematological investigation of patients with neutropenia. As there have been occasional reports concerning hair colourants and haemopoietic disorders¹, we decided to investigate the long term effects of two 'semipermanent hair colourants' which came to our attention during routine questioning. A more extensive range of colourants (coded A–L) have been tested for mutagenic activity in bacteria. The effects of two constituent dyes have also been tested in bacteria and in human lymphocytes.

The colourants tested initially were J, one of a variety of proprietary preparations containing nitrophenylenediamines, and K, containing an azo-dye metal derivative and an amino-nitrophenol. Both contain detergent and perfume. Their effects

are being examined by repeated applications in aqueous acetone solution to the skin of A and DBA strain mice. Although topical applications were intended to provide a reasonable approximation to human usage, the mice groom themselves after treatment so that considerable amounts of colourant are probably ingested orally in addition to any that may be absorbed through the skin. No lesions have occurred on the treated skin.

In the DBA mice malignant tumours have, however, occurred so far in 5 out of 48 mice treated with compound J (26, 37, 41, 51, 61 weeks), two out of 32 mice treated with K (41, 47 weeks), and none of 32 controls. In the A strain mice malignant tumours have occurred in 4 out of 52 mice treated with J (48, 55, 57, 59

Table 1 Mutagenicity assays of 11 hair colourants in *S. typhimurium* TA1538, with or without liver-microsomal activation

Test material	Induced <i>his</i> ⁺ revertant colonies per plate			
Benzo(a)pyrene (5 µg per plate)	—(S-9) 19 +(S-9) 188			
Hair colourant	Diluted (×10)		Diluted (×100)	
	—(S-9)	+(S-9)	—(S-9)	+(S-9)
A	7	0	7	0
B*	975	29	155	0
C*†	307	345	34	84
D*	807	614	157	57
E	18	0	13	0
F	0	0	8	0
G*	144	—	44	—
H	5	0	32	0
J*	1071	208	112	33
K*	15	388	28	228
L*	956	875	195	144

*Denotes levels of mutation considered significant in this assay system.

†Oxidising dye containing toluenylenediamine.

Each hair colourant was diluted tenfold (v/v) in deionised water, filter-sterilised and a further tenfold dilution in sterile water was prepared. Samples (0.1 ml) of each dilution were assayed for their ability to induce reversion to *his*⁺ in *S. typhimurium* TA1538 in the presence or absence of benzo(a)pyrene-induced rat-liver supernatant (S-9 mix) by the methods described by Ames *et al.*². Benzo(a)pyrene was used as a positive control for the metabolic activity of the S-9 mix. Plates were scored for revertant colonies after 3 d incubation at 37 °C. Values shown are induced *his*⁺ revertants per plate (mean of two plates) after subtraction of spontaneous revertants (12 per plate without S-9, 45 per plate with S-9).

weeks), 3 out of 32 mice treated with K (38, 38, 72 weeks), and also in 3 out of 32 controls (61, 75, 77 weeks). All the tumours have been of the lymphoid system. In three mice treated with J, these were thymic lymphosarcomas but in the other animals the tumours were most commonly malignant lymphomas involving the spleen with infiltration of the liver and other organs. With most of the mice still on test, it is too early to evaluate the significance of these observations, especially in view of the well-known role of oncogenic viruses in the causation of malignant lymphomas in mice. It was, however, the recognition of these tumours at a relatively early age in the treated animals which stimulated further studies on the mutagenicity of these colourants.

Carcinogenicity tests, particularly with weak carcinogens, are prolonged and expensive. There is great interest in short term tests, which depend on the reasonable correlations observed between carcinogenicity and mutagenicity², for screening substances present in the environment. We have tested colourants J and K, and nine similar products, for mutagenicity in bacteria. The organisms used were *Escherichia coli* WP2, WP2 *uvrA*, and WP2 *exrA*, all of which are *trp*[−] and revert to *trp*⁺ by base substitution³; *Salmonella typhimurium* TA1535, TA1537, and TA1538, all of which are *his*[−], *uvrB*, and have defective lipopolysaccharide coats which render them highly permeable to many mutagenic carcinogens. TA1535 is a base-substitution mutant, and TA1537 and TA1538 are both frame-shift mutants⁴.

Table 2 Mutagenicity of 2-nitro-*p*-phenylenediamine and 4-nitro-*o*-phenylenediamine in *S. typhimurium* TA1538 with or without liver-microsomal activation

Dose applied	Induced <i>his</i> ⁺ revertants per plate			
	5 µg per plate		50 µg per plate	
	-(S-9)	+(S-9)	-(S-9)	+(S-9)
Test compound				
2-NPPD	43	27	335	213
4-NOPD	133	188	883	727

A pour-plate technique was used for all mutagenicity assays. For the *E. coli* series, the top agar contained 5% nutrient broth, 5 µg ml⁻¹ tryptophan, 0.6% NaCl and 0.6% agar. The *Salmonella* series was assayed as described in Table 1. Vogel-Bonner MEM⁵, with 1.5% agar and 2% glucose, was used as bottom agar in all assays.

Negative results were obtained in those bacteria which revert by base-pair substitution (*E. coli* WP2 series, and *S. typhimurium* TA1535). Seven of the eleven colourants were strongly mutagenic in *S. typhimurium* TA1538 (Table 1), and of these, two (D and J) were weakly mutagenic in TA1537. Six colourants (B, C, D, G, J and L) did not require microsomal activation for mutagenic activity, and B and J were markedly less mutagenic in the presence of the S-9 liver fraction. K, however, was mutagenic only when activated by the S-9 fraction.

The two dyes, 2-nitro-*p*-phenylenediamine (2-NPPD) and 4-nitro-*o*-phenylenediamine (4-NOPD); (purity >97%), present in colourant J (both from Aldrich, Wembley, UK) were tested without purification, and found to be mutagenic in TA1538 (Table 2) and weakly mutagenic in TA1537. 4-NOPD was about three times more potent than 2-NPPD at equivalent doses. Microsomal activation was not required for this activity. Ames⁶ has also found 4-NOPD to be mutagenic in *Salmonella*.

To determine whether the mutagenic colourants contained either or both of these dyes, each colourant was fractionated using thin-layer chromatography on silica gel (Polygram Sil N-HR/UV₂₅₄; Camlab, Cambridge) in diethylether-petroleum ether (4:1) using 2-NPPD and 4-NOPD as markers. This solvent resolved three to ten coloured spots from colourants B, C, D, G, J and L. A strip (1 cm wide) was cut from each chromatogram and the spots, and the colourless areas between, were cut out and assayed for mutagenicity by plating with TA1538. This technique showed that each of these colourants contained fractions of high mutagenicity corresponding in *R_f* and colour to 2-NPPD and/or 4-NOPD. In some cases, mutagenic activity was also associated with colourless areas near the origin. The identity of the mutagenic agent(s) in

colourant K, which requires metabolic activation, has not been determined.

Many carcinogens and mutagens are known to be chromosome-breaking agents. When the colourants J and K were added to cultures of human peripheral blood lymphocytes⁷, serious toxicity, presumably caused by detergent, was noted. No chromosome damage was observed when dilutions high enough to avoid this toxicity were used. A series of experiments using 4-NOPD at concentrations up to 100 µg ml⁻¹ failed to show any chromosome damage in cultures incubated for 48 and 72 h when the dye was added at the start of incubation, or at 24 h or 4 h before collecting. Similar experiments with 2-NPPD at concentrations between 50 µg ml⁻¹ and 100 µg ml⁻¹, however, showed a considerable number of chromosome and chromatid gaps and breaks (Table 3). At 100 µg ml⁻¹, mitotic delay and toxicity were evident, but it was still possible to show that up to 45% of the cells contained damaged chromosomes. Cultures treated with lower concentrations of 2-NPPD did not differ significantly from controls.

Although a significant degree of carcinogenicity in a cosmetic preparation would undoubtedly be regarded as quite unacceptable, the human relevance of mutagenic activity is much harder to assess, especially when there are additional uncertainties about amounts of constituents absorbed into the body. The short term toxicity of *p*-phenylenediamine in man is well established¹. The nitrophenylenediamines are, however, less well studied and those we purchased carried a warning that their toxicological properties have not been fully investigated and that their handling or use may be hazardous. They are, nevertheless, widely used in hair colourants, some of which we find to be mutagenic and possibly carcinogenic. Our observations suggest a possible long term hazard and indicate the need for comprehensive toxicological evaluation of hair colourant constituents.

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Table 3 Effect of 2-nitro-*p*-phenylenediamine on cultured human lymphocytes

	Total cells examined	Number of cells with aberrations		
		B cells	C cells	Total abnormal
2-NPPD present from time zero (µg ml ⁻¹)				
75	100	10	1	11
50	100	8	1	9
25	100	5	0	5
2-NPPD present for 24 h before harvesting (µg ml ⁻¹);				
75	100	21	2	23
50	100	17	0	17
25	100	2	1	3
Control	100	3	2	5

Human peripheral blood lymphocyte cultures were collected at 48 h. Cells were stained with orcein and scored for aberrations but not fully analysed. The classification of Buckton and Pike⁸ was used. B cells have chromosome or chromatid gaps or breaks only, whereas C cells have stable or unstable chromosome rearrangements

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¹ Hunter, D., *The Diseases of Occupations*, fourth ed., 571-573 (English Universities Press, London, 1969).

² Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 2281-2285 (1973).

³ Bridges, B. A., Dennis, R., and Munson, R. J., *Genetics*, **57**, 897-908 (1967).

⁴ Ames, B. N., Lee, F. D., and Durston, W. E., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 782-786 (1973).

⁵ Vogel, H. J., and Bonner, D. M., *J. biol. Chem.*, **218**, 97-106 (1956).

⁶ Ames, B. N., *Proc. 11th Int. Cancer Congr.* (Florence, October, 1974).

⁷ Moorhead, P. S., Nowell, P. C., Mellman, N. J., Battips, D. M., and Hungerford, D. A., *Expt Cell Res.*, **20**, 613-616 (1960).

⁸ Buckton, K. E., and Pike, M. C., *Int. J. radiat. Biol.*, **8**, 439-452 (1964).

Helper function of T7 protein kinase in virus propagation

A PROTEIN kinase, the genetic information for which is phage-coded, is induced after infection of *Escherichia coli* with either phage T7 or T3 (ref. 1). The protein kinase is important in the regulation of gene expression in that it inhibits the initiation of transcription by *E. coli* RNA polymerase and in that it restricts host-dependent transcription of the T7 genome to the early region². T7 protein kinase phosphorylates the β' subunit of *E. coli* RNA polymerase (this action provides an explanation for the effects on transcription) and other *E. coli* proteins^{1,2}.

From the above considerations, one would have expected that the absence of protein kinase would have serious effects on phage development. The contrary was found, however, and the burst size or latent period in infection did not depend on the presence of protein kinase^{1,2}. Furthermore, one widely distributed T7 strain and its many derivatives was found to lack the gene for protein kinase². Yet no growth disadvantages of these phages to protein kinase-synthesising T7 strains was observed during many years of laboratory cultivation. If the protein kinase gene was not needed, what made it survive during evolution?

The results presented here demonstrate that protein kinase is in fact required for phage development in suboptimal growth conditions. In those conditions, the absence of protein kinase can lead to a very low or nonexistent burst size. The two growth conditions which we found to date and which display an effect of a protein kinase deficiency are propagation in poor media, and propagation at elevated temperature.

The effects of poor media were demonstrated for the development of T7 in various *K12* strains. Protein kinase-deficient mutants of phage T7 (T7 K^-) gave only few, if any, progeny

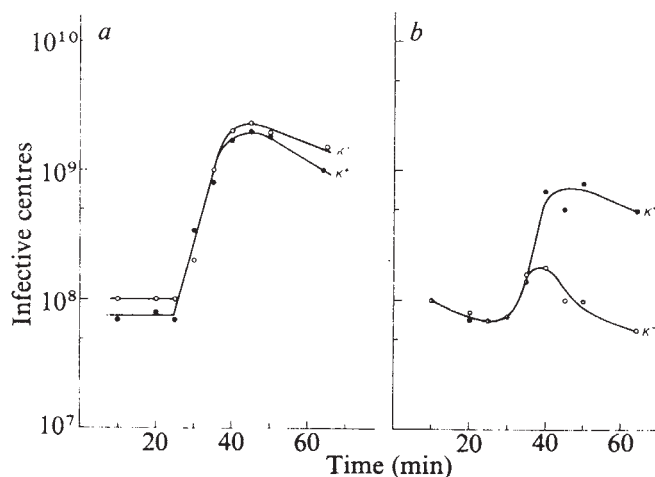


Fig. 1 Development of T7 with and without protein kinase in *E. coli K12* grown in minimal medium. *a*, *E. coli K12* 514 and *b*, *E. coli B*₅₋₁ were grown at 30 °C in M9 medium (Na₂HPO₄, 2 H₂O (7.2), KH₂PO₄ (3), NaCl (0.5), NH₄Cl (1.0); and 0.4% glucose). At an optical density (600 nm) of 0.2, the cells were infected with either T7 K^+ (●) or T7 K^- (○) (isogenic pair as described in ref. 2) at a multiplicity of infection of 10. To remove non-adsorbed virus, antibodies were added at 5 min after infection. At 10 min after infection, part of the infected cells were diluted 1:10³, 1:10⁴, 1:10⁵ and 1:10⁶ and incubation at 30 °C continued. Aliquots (0.1 ml) of the dilutions were plated with indicator bacteria at various times. The undiluted part was used for the pulse-label experiment shown in Fig. 2. Surviving cells were plated at 3 min after infection.

Table 1 Requirement of T7 protein kinase for propagation in minimal medium

Host	Infecting phage	
	T7 K^+	T7 K^-
M9 medium		
<i>E. coli K12</i>		
514	9	0.6
W1655	1.5	0.15
C600	0.6	0.03
ED3736	13	3
YC3272	2	0.25
803	0.6	0.06
<i>E. coli B</i>		
B ₈₋₁	16	14
BB	17	17
O11	1	2
<i>E. coli C</i>		
HF4704	1	0.6
HF4714	0.4	0.2
Tryptone broth		
B ₈₋₁	112	80
514	25	29

Various *E. coli K12* strains and *E. coli B* strains were grown either in M9 medium (Fig. 1) or in tryptone broth (1% tryptone, 0.5% NaCl) and infected with T7 K^+ or T7 K^- , respectively (multiplicity of infection 10). Survivors were plated 3 min after infection. Infected cells were determined by the difference between input cells and survivors. At 5 min, non-adsorbed T7 were inactivated by antiserum. At 10 min, cells were diluted 1:1,000 and the number of progeny virus determined at various times thereafter. Burst size per infected cell was calculated and listed.

Strains used have the following properties: W1655 (*E. coli K12*): F_λ33-43, met⁺, RC⁻; C600 (*E. coli K12*): B₁⁻, thr⁻, leu⁻, su⁺, Φ80^R; ED3736 (*E. coli K12*): his⁻, trp⁻, lac⁻, T6^R, str^R, gel⁻, su⁻; IC3272 (*E. coli K12*): F⁻, lac_λ×74, gal⁻, mal⁻, λcrp⁺, λ^R, T6^R, T1^R, B₁⁻, his⁻, trp⁻, lys⁻, str^R, su⁻; 514 (*E. coli K12*): F⁻, lac_λ, trp⁻, str^R; 803 (*E. coli K12*): gal⁻, met⁻, lac⁻, r_K⁻, m_K⁻; B₈₋₁ (*E. coli B*): uvrA⁻, su⁻; BB: su⁺ derivative of *E. coli B*; O11: su⁻ derivative of *E. coli B*; HF4704 (*E. coli C*): ΦX174⁺, su⁻, trp⁻; HF4714 (*E. coli C*): ΦX174⁺, su⁺, trp⁻.

phages, when used to infect *E. coli K12* grown in minimal medium, whereas the wild type phage (T7 K^+) was propagated (Table 1). In rich medium, such as tryptone broth or tryptone yeast broth, T7 K^- gave as many infective centres as did T7 K^+ (Table 1). The property to select against protein kinase-deficient T7 in poor media was a general feature of all *K12* strains tested (Table 1), whereas other *E. coli* strains, such as *E. coli B* and *E. coli C*, propagated T7 K^+ or K^- with approximately equal efficiency regardless of whether they were grown in tryptone broth or minimal medium (Table 1). Since *E. coli B* is the most commonly used strain for propagating T7, the effects of kinase would not have been detected.

The latent periods were identical with T7 K^+ and with T7 K^- when grown on *E. coli K12* minimal medium (Fig. 1). The reduction in T7 K^- progeny reflects the reduction in burst size per phage-developing cell (Fig. 1). In some strains this was less than one—the initial number of infective centres even decreasing with time, indicating that some fraction of cells did not develop any virus at all unless they were saved early on by plating on rich medium (data not shown).

Associated with the inefficient development of protein kinase-deficient T7 in *E. coli K12* is a reduction in late viral protein synthesis at times beyond 14 min after infection. In pulse experiments with ¹⁴C-labelled amino acids and subsequent analysis of the proteins in slab-gel electrophoresis the products of the T7 late genes 16, 12, 15, 17, 8, and 10 were hardly detectable during 16–20 min, whereas the control experiment with wild type T7 showed a maximal rate of synthesis of these proteins at this time (Fig. 2). In *E. coli B*₈₋₁ (with the best burst size) late protein synthesis continues even further (Fig. 2). Thus, burst size seems to be directly related to the efficiency of late protein synthesis.

T7 K^- bacteriophage particles were less stable than T7 K^+ progeny after growth in *E. coli K12* in minimal medium. As indicated in the one-step growth curve (Fig. 1), 60% of T7 K^- were inactivated at 30 °C within 20 min. The instability of T7 K^- progeny cannot, however, account for all the difference

between T7 K^+ and K^- ; in an experiment in which newly-formed phages were allowed to adsorb to cells, the difference between the number of infective centres of T7 K^+ and T7 K^- was still apparent and similar to that shown in Fig. 1.

T7 protein kinase seems to promote late phage protein synthesis in *E. coli* K12 grown in suboptimal conditions. A similar but weaker effect has also been observed in *E. coli* B (barely visible in Fig. 2a, but see evaluation in Fig. 6, ref. 2) but did not result in a measurable decrease in burst size. For both K12 and B, large proteins were more sensitive to the lack of support from protein kinase than small proteins.

Growth of any *E. coli* host at elevated temperature also reduced the yield of protein kinase-deficient T7 and at 45 °C the burst size dropped to virtually zero (Fig. 3). This inhibition occurred in rich and poor media. The burst of T7 wild type was only slightly decreased. The difference in detectable phage

Fig. 2 The efficiency of late T7 protein synthesis during virus propagation in suboptimal conditions. Aliquots (0.2 ml) of infected cells from the experiments in Figs 1 and 3 (from the non-diluted culture) were removed at various times and pulse-labelled with ^{14}C amino acids (5 μCi ^{14}C protein hydrolysate) according to ref. 2. The samples from the experiment shown in Fig. 1 were pulse-labelled at 30 °C for 4 min (a), those of Fig. 3 at 45 °C for 1 min (b). After the pulse, incubation was continued with an excess of cold amino acids for 1 min; the cells were then collected, dissolved in sodium dodecyl sulphate buffer and the proteins resolved by polyacrylamide slab-gel electrophoresis (10B20% linear gradient). Autoradiograms of the dried gels are shown. The numbers on top of each position represent the beginning of pulse. Some T7 protein bands are marked by the numbers of the corresponding genes.

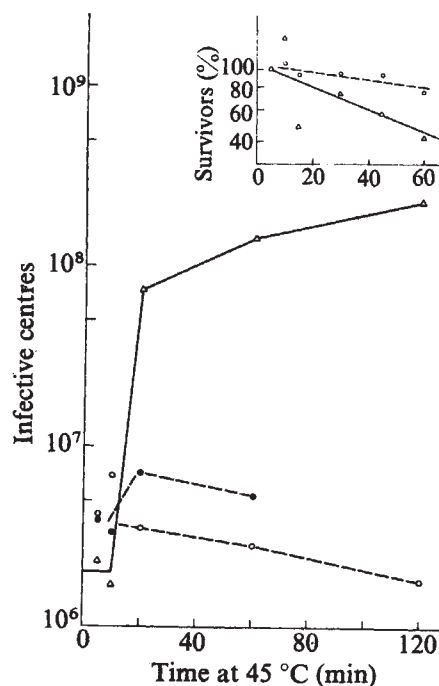
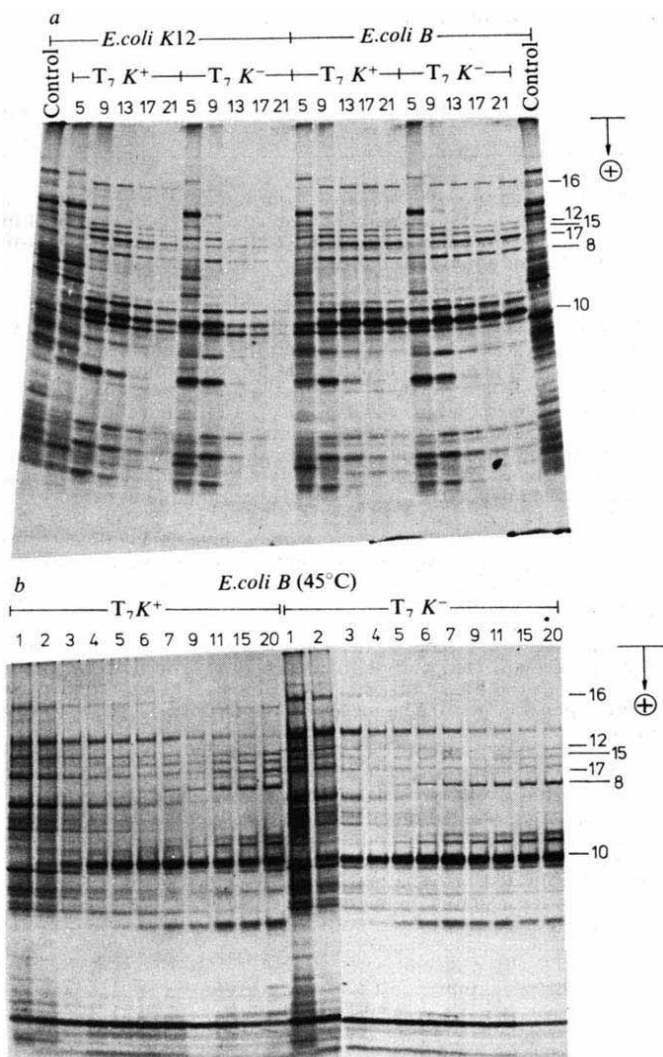


Fig. 3 Development of T7 K^- in *E. coli* B at elevated temperature. *E. coli* B_{S-1} were grown in M9 medium at 37 °C. At an optical density of 0.2 (600 nm) part of the culture was shifted to 45 °C, the other 44 °C. After an additional 5 min, 0.5 ml aliquots were infected with T7 K^+ (Δ, 45 °C; 44 °C not shown) or T7 K^- (○, 45 °C; ●, 44 °C) respectively, all at an approximate multiplicity of infection of 0.1. At 3 min, cells were diluted 1:10⁴ into medium of the appropriate temperature and aliquots plated with indicator bacteria at various times (the plates were kept at 30 °C). The insert shows the survival of T7 K^+ (Δ) and T7 K^- (○) at 45 °C.

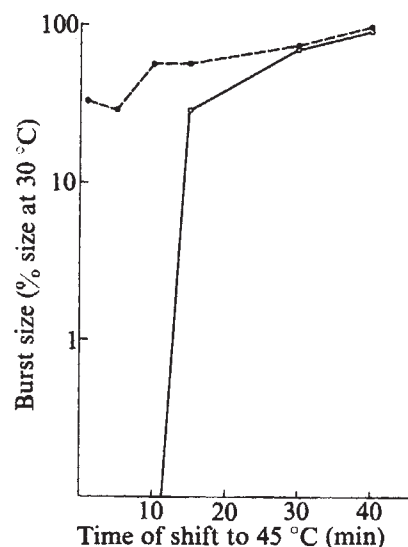


Fig. 4 Requirement for T7 protein kinase at 45 °C. Kinetics of occurrence of the heat-sensitive step. A culture of *E. coli* B_{S-1} in tryptone broth at 30 °C was infected with T7 K^+ or T7 K^- , respectively, at an absorbance of 0.2 (600 nm) and a multiplicity of infection of 0.1. At various times after infection, aliquots were diluted 1:10⁴ into tryptone broth kept at 45 °C. Subsequent phage development was monitored and the burst size per infected cell determined. The burst size (% size at 30 °C) was plotted. ●, T7 K^+ ; ○, T7 K^- (data for 1–10 min were below 0.1%).

cannot be attributed to a difference in heat stability, as protein kinase-deficient phages were more heat stable at 45 °C than the wild type (Fig. 3), (the kinase mutation is the result of a small deletion and deletions have been found to be more heat stable³⁻⁵).

The effect of heat on viral growth occurs late in virus development. A shift from 30 to 45 °C reduced the virus output even when applied as late as 15 min after infection (Fig. 4). At that time, most DNA synthesis and late protein is already completed. In agreement with this observation the kinase mutation did not have any effects on the rate of synthesis of early and late proteins at 45 °C (Fig. 2). Although late T7 protein synthesis induced by T7 K⁻ was normal, no intracellular premature phage (not shown) and no release of mature phage was observed in these conditions (Fig. 3).

Our data suggest that the protein kinase was necessary for different steps in phage production at 45 °C and for growth in minimal medium with K12 strains. At 45 °C, protein kinase stabilised an essential process late in viral development cycle, whereas in K12 grown in M9 medium, protein kinase was needed for sufficient accumulation of late proteins. Experiments with T3 phage carrying a protein kinase mutation and with T3 wild type yielded similar results.

Our experiments indicate that the T7 and T3-specific protein kinase is required for the development of phage in suboptimal conditions. Outside the laboratory, where growth conditions are likely to be suboptimal, the protein kinase gene may have some survival value. Indeed, T7 and T3 phage collected from various fields all induced protein kinase (R. Goslich, unpublished). Thus, the natural environment has conserved the gene for protein kinase during evolution.

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¹ Rahmsdorf, H. J., et al., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 586 (1974).

² Ponta, H. et al., *Molec. gen. Genet.*, **134**, 281 (1974).

³ Rubenstein, I., *Virology*, **36**, 356 (1968).

⁴ Parkinson, J. S., and Huskey, R. J., *J. molec. Biol.*, **56**, 369 (1971).

⁵ Studier, F. W., *Science*, **176**, 367 (1972).

Seryl-tRNA synthetase and activation of the carcinogen 4-nitroquinoline 1-oxide

THE carcinogenic and mutagenic compound, 4-nitroquinoline 1-oxide (4NQO) and its reduced metabolite, 4-hydroxyaminoquinoline 1-oxide (4HAQO) bind covalently to cellular macromolecules such as nucleic acid and protein¹⁻⁷. As 4HAQO rarely binds to nucleic acid in buffer solutions, it was suggested that a metabolic activation of 4HAQO is involved in the *in vivo* reaction. The presence of an activating enzyme has been demonstrated in extracts of rat ascites hepatoma cells and bakers' yeast^{8,9}. The enzyme activates 4HAQO to bind to nucleic acid or protein in the presence of ATP, L-serine and Mg²⁺ (ref. 9). The nucleic acid adducts formed in the enzyme system show essentially the same chromatographic patterns as those of the purine adducts formed *in vivo*^{4,6,8}. Moreover, the DNA lesions caused by 4HAQO binding in the *in vitro* enzyme system, like those lesions formed *in vivo*, can be repaired by the excision repair mechanism in *Bacillus subtilis*¹⁰. We have now purified 4HAQO-activating enzyme from bakers' yeast and report here that this enzyme is seryl-tRNA synthetase.

The enzyme was purified from commercial bakers' yeast to near homogeneity (Table 1, detailed procedures will be reported elsewhere). As reported previously, 4HAQO-activating enzyme requires ATP and L-serine for its binding activity⁹ as well as Mg²⁺ and an sulphhydryl protective reagent for maximal activity⁸. To characterise the reaction, we have analysed the *in vitro* reaction products by paper electrophoresis. As shown in Fig. 1, ³H-AMP and ³²P-pyrophosphate were detected among the reaction products when ³H-ATP and γ-³²P-ATP, respectively, were initially present. No significant conversion of L-serine was observed however. The mode of ATP degradation and the requirement for L-serine suggest that the reaction may be catalysed by seryl-tRNA synthetase similar to the well known amino acid activation catalysed by aminoacyl-tRNA synthetase in which degradation of ATP to AMP and pyrophosphate takes place if an appropriate amino acid is present.

After testing the purified enzyme for seryl-tRNA synthetase activity, we found that this activity was associated with 4HAQO-activating enzyme throughout the purification steps (Table 1). These two activities could not be separated by chromatography on DEAE-cellulose or Sephadex G-200 (Fig. 2) or by centrifugation on a sucrose density gradient. Both activities showed the

Table 1 Relative rate of 4HAQO-binding and seryl-tRNA synthesising activities during purification of 4HAQO-activating enzyme

Purification step	4HAQO binding*	Seryl-tRNA synthesis*
Autolysate	0.5	0.6
Acetone powder	1.0	1.0
Phosphocellulose	1.5	1.6
First ethanol precipitation	6.1	4.6
Second ethanol precipitation	34.6	28.8
DEAE-cellulose	97.8	105.4
DEAE-Sephadex	206	206
Hydroxylapatite	487	457

*Relative rate of specific activities.

Acetone-dried powder prepared from the autolysate of yeast⁹ was extracted with 0.02 M potassium phosphate, pH 6.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). The extract clarified by centrifugation was passed through a phosphocellulose column, and enzyme in the unadsorbed fractions was precipitated by the addition of Mg(OAc)₂ (10 mM) and ethanol (0.3 volumes) at -20 °C. Enzyme was dissolved in buffer A containing 10 mM Mg(OAc)₂, treated with 30% ethanol at 37 °C for 10 min, and the precipitate was extracted with 0.02 M potassium phosphate, pH 6.5, containing 1 mM EDTA, 1 mM Mg(OAc)₂, 1 mM 2-mercaptoethanol and 5% glycerol (buffer B). The enzyme solution was loaded on a DEAE-cellulose column, eluted with 0.25 M potassium phosphate, pH 6.5, and dialysed against buffer B. The enzyme preparation was then chromatographed on a DEAE-Sephadex A-50 column eluting with a potassium phosphate linear gradient from 0.05 to 0.35 M, pH 6.5, containing 1 mM EDTA, 1 mM Mg(OAc)₂, 1 mM 2-mercaptoethanol and 5% glycerol (buffer C). The enzyme preparation was then chromatographed on a DEAE-Sephadex A-50 column eluting with a potassium phosphate linear gradient from 0.02 to 0.3 M, pH 7.0, in the presence of 10% glycerol and 1 mM each of EDTA, Mg(OAc)₂ and 2-mercaptoethanol. Fractions containing enzyme were collected, dialysed against buffer C and stored at -20 °C. Activities in the final step were 0.43 μmol 4HAQO bound per mg protein per min and 0.53 μmol seryl-tRNA formed per mg protein per min. The reaction mixture (0.1 ml) for the 4HAQO-binding assay contained 1 mM ³H-4HAQO (1.5 Ci mol⁻¹), 3 mM ATP, 10 mM L-serine, 3 mM Mg(OAc)₂, 1 mM DTT, 100 μg ml⁻¹ bovine serum albumin, 20 mg ml⁻¹ yeast RNA, 50 mM Bicine-KOH, pH 9.0, and enzyme. After incubation at 30 °C for 10 min, the reaction was stopped by the addition of sodium dodecyl sulphate solution followed by phenol extraction⁸. RNA was precipitated by the addition of ethanol, collected on Whatman GF/C paper disks and washed with cold TCA. Radioactivity remaining was measured in a liquid scintillation counter. The assay of seryl-tRNA synthetase activity was carried out in 0.05 ml containing 0.1 mM U-¹⁴C-L-serine (2.0 Ci mol⁻¹), 3 mM ATP, 10 A₂₆₀ units ml⁻¹ tRNA^{ser} from bakers' yeast (0.57 nmol serine A₂₆₀ unit⁻¹) 10 mM Mg(OAc)₂, 1 mM DTT, 50 mM Bicine-KOH, pH 9.0, and enzyme. Incubations were carried out at 30 °C for 10 min and stopped by pipetting 40 μl of the reaction mixture on Whatman 3MM paper disks which were washed and counted¹¹.

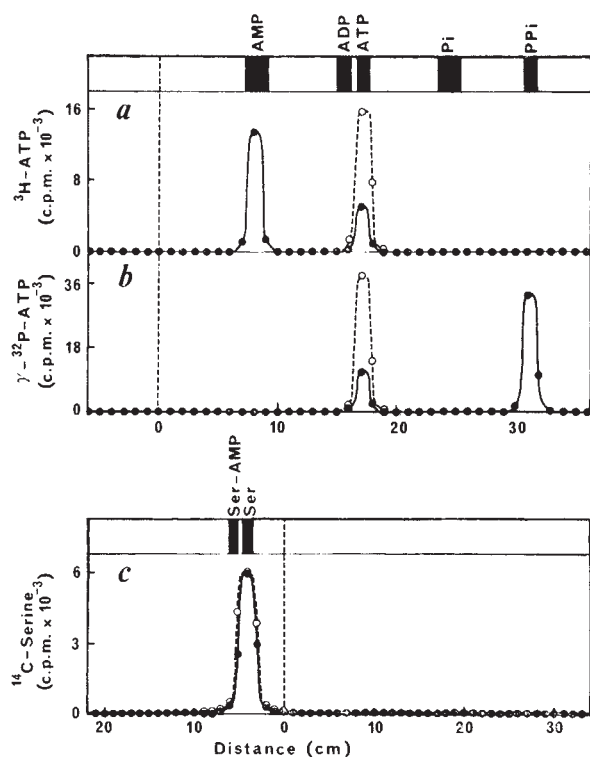


Fig. 1 Electrophoretic analysis of the reaction products of the 4HAQO-activating reaction. After the reaction electrophoresis was carried out on Whatman 3MM paper in 0.05 M sodium citrate, pH 3.9, at 37 V cm⁻¹ for 90 min, the paper was cut and radioactivity was measured by liquid scintillation⁴. *a*, 2-³H-ATP (0.2 mM; 125 Ci mol⁻¹; Radiochemical Centre, Amersham) was incubated at 30 °C for 15 min in the reaction mixture (0.1 ml) containing 10 mM L-serine, 1 mM dithiothreitol (DTT), 5 mM Mg(OAc)₂, 50 mM sodium borate, pH 9.0, 10 µg ml⁻¹ enzyme in the presence (●) or absence (○) of 1 mM 4HAQO; *b*, γ-³²P-ATP (0.2 mM; 6.25 Ci mol⁻¹; Radiochemical Centre, Amersham) incubated as above *c*, U-¹⁴C-L-Serine (0.01 mM; 130 Ci mol⁻¹; Daiichi, Tokyo) incubated in the reaction mixture in the presence (●) or absence (○) of 4HAQO as above except 2 mM ATP was added and unlabelled L-serine was omitted. Pi, Orthophosphate; PPI pyrophosphate.

same stability against heat inactivation. The specific activity of the purified enzyme for seryl-tRNA synthesis was comparable with that of highly purified yeast seryl-tRNA synthetase as reported by Heider *et al.*¹¹. From these results we conclude that seryl-tRNA synthetase also catalyses the binding of 4HAQO to nucleic acid. Figure 3 summarises our proposed model for the enzyme-mediated reaction scheme of 4HAQO and nucleic acid. We assume, as is the case with other aminoacyl-tRNA synthetases (for example, ref. 12), that seryl-AMP is made on the

Table 2 Binding of 4HAQO to RNA by seryl-tRNA synthetase and by seryl-AMP

	Extent of binding (nmol 4HAQO)
Enzymic reaction	
Complete	3.54
Minus enzyme	0.09
Non-enzymic reaction	
Complete	6.55
Minus seryl-AMP	0.09

Enzymic 4HAQO binding was carried out in the reaction mixture as in Table 1 except that 2.5 mg ml⁻¹ yeast RNA and 60 µg ml⁻¹ purified enzyme were used. The reaction mixture (0.1 ml) for non-enzymic binding contained 1 mM ³H-4HAQO (1.5 Ci mol⁻¹), 2.5 mg ml⁻¹ yeast RNA, 1 mM DTT, 50 mM Bicine-KOH, pH 8.0, and 0.4 mM seryl-AMP which was synthesised according to the method of Berg¹³. Both reactions were carried out at 30 °C for 10 min and other conditions of assay were as described in Table 1.

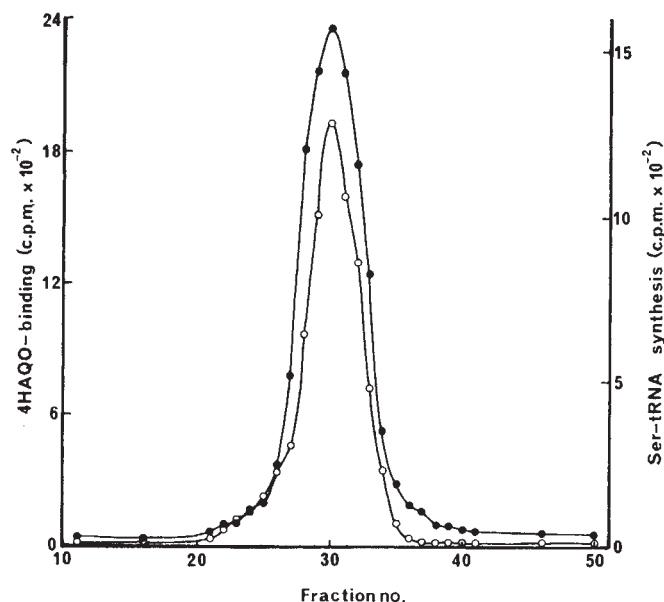
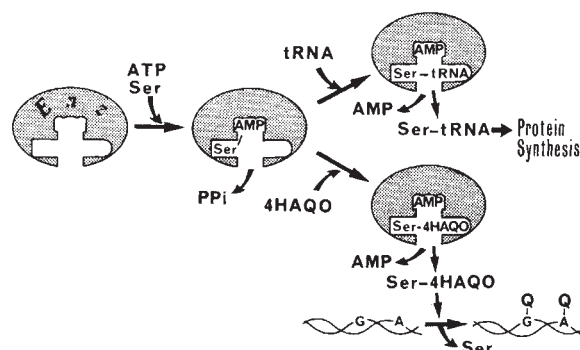


Fig. 2 Chromatographic elution pattern of 4HAQO binding (●) and seryl-tRNA-synthesising (○) activity from a Sephadex G-200 column (80 × 1.1 cm) loaded with 800 µg the purified enzyme, equilibrated and eluted with buffer C. The measurements of the enzyme activities were carried out as in Table 1.

seryl-tRNA synthetase molecule. As the addition of 4HAQO to this reaction mixture results in the consumption of ATP and the concomitant release of AMP and pyrophosphate, as in the case of the addition of tRNA (Fig. 1), we further assume that 4HAQO reacts with seryl-AMP bound on the enzyme, producing seryl-4HAQO, analogous to the way in which the addition of tRNA results in the formation of seryl-tRNA. This assumption is supported, though not proved, by the finding that the binding of 4HAQO to nucleic acid can be activated by seryl-AMP in the absence of the enzyme (Table 2). In this system, however, seryl-AMP hydrolyses so rapidly that the detailed kinetics of the reaction cannot be examined. Other kinds of aminoacyl-AMP, such as leucyl-AMP, also activate 4HAQO. The activated 4HAQO in the present system, either enzymic or non-enzymic, reacts with purines but not with pyrimidines giving rise to adducts. The activated 4HAQO is too unstable to be isolated and its structure determined.

It has been noted previously⁹ that the 4HAQO-binding reaction including yeast preparation is stimulated only by L-serine, whereas the reaction with rat hepatoma cell prepara-

Fig. 3 A proposed mechanism of 4HAQO activation. In the first process of the reaction seryl-AMP (Ser-AMP) is formed on seryl-tRNA synthetase (Enz) from L-serine (Ser) and ATP. In the physiological pathway, tRNA then accepts the serine residue on the enzyme to form seryl-tRNA (Ser-tRNA) which is utilised in protein synthesis. If 4HAQO is present, however, it may be acylated by the seryl-AMP-enzyme complex. Seryl-4HAQO (Ser-4HAQO), a possible reactive species, may then introduce quinoline groups (Q) into nucleic acid at guanine (G) and adenine (A) residues (M.T. and M.T., unpublished).



tion is stimulated by both L-serine and L-proline. This suggests that 4HAQO may be activated by both seryl- and prolyl-tRNA synthetases in rat tissues. Aminoacyl-tRNA synthetases which are capable of 4HAQO-activation, may have a unique conformation enabling them to aminoacylate the N-hydroxyl group of the carcinogen. Carcinogenic aromatic amines and nitro compounds undoubtedly require metabolic activation for their reactivity *in vivo*. The first activation step of these compounds seems to be formation of N-hydroxyderivatives, and it has been suggested that the final activation step is N-hydroxyesterification¹⁴. Seryl-tRNA synthetase and some other species of aminoacyl-tRNA synthetase may participate *in vivo* in the activation of not only 4HAQO but also some other N-hydroxy compounds through their aminoacylating capacity.

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- ¹ Okabayashi, T., and Yoshimoto, A., *Chem. pharm. Bull., Tokyo*, **10**, 1221–1226 (1962).
- ² Sugimura, T., Okabe, K., and Endo, H., *Gann*, **56**, 489–501 (1965).
- ³ Tada, M., Tada, M., and Takahashi, T., *Biochem. biophys. Res. Commun.*, **29**, 469–477 (1967).
- ⁴ Tada, M., and Tada, M., *Chemico-biol. Interactions*, **3**, 225–229 (1971).
- ⁵ Matsushima, T., Kobuna, I., and Sugimura, T., *Nature*, **216**, 508 (1967).
- ⁶ Ikegami, S., Nemoto, N., Sato, W., and Sugimura, T., *Chemico-biol. Interactions*, **1**, 321–330 (1969/70).
- ⁷ Andoh, T., Kato, K., Takaoka, T., and Katsuta, H., *Int. J. Cancer*, **7**, 455–467 (1971).
- ⁸ Tada, M., and Tada, M., *Biochem. biophys. Res. Commun.*, **46**, 1025–1032 (1972).
- ⁹ Tada, M., and Tada, M., *Gann*, **65**, 281–284 (1974).
- ¹⁰ Tanooka, H., Tada, M., and Tada, M., *Chemico-biol. Interactions*, **10**, 11–18 (1975).
- ¹¹ Heider, H., Gottschalk, E., and Cramer, F., *Eur. J. Biochem.*, **20**, 144–152 (1971).
- ¹² Loftfield, R. B., in *Progress in Nucleic Acid Research and Molecular Biology* (edit. by Davidson, J. N., and Cohn, W. E.), **87–128** (Academic, New York, 1972).
- ¹³ Berg, P., *J. biol. Chem.*, **233**, 608–611 (1958).
- ¹⁴ Miller, J. A., *Cancer Res.*, **30**, 559–576 (1970).

K region benzo(α)pyrene-4,5-oxide is conjugated by homogeneous glutathione S-transferases

We report direct evidence that conjugation with glutathione (GSH) is a significant mechanism for detoxification of the epoxides of polycyclic aromatic hydrocarbons. We believe that the level of the enzymes may be directly relevant to the carcinogenic potential of the hydrocarbons, which are common pollutants¹. They are metabolised by microsomal mixed function oxygenases and associated enzymes, to detoxified as well as carcinogenic forms^{2–4}. One of the metabolic products of benzo(α)pyrene is the 'K' region epoxide, benzo(α)pyrene-4,5-oxide. Such epoxides bind to macromolecules, are mutagenic and may be the carcinogenic forms of the polycyclic aromatic hydrocarbons⁵. Benzo(α)pyrene-4,5-oxide is converted enzymatically either to the dihydrodiol by hydrolysis⁶ or to a thioether by conjugation with GSH⁷. The glutathione S-transferases (EC 2.5.1.18) detoxify many compounds^{8,9} including alkyl epoxides¹⁰ and arene oxides^{11–12}. Now that pure glutathione S-transferases are available (five rat^{10,12,13} and five human, K. Kamisaka, W. H. H., J. N. K., I. M. Arias and W. B. J., unpublished), we have been able to evaluate directly the extent to which these enzymes can produce GSH conjugates of the polycyclic aromatic oxide and, presumably, detoxify it.

Incubation of each of the enzymes with benzo(α)pyrene-4,5-oxide and GSH in standard assay conditions⁷ always resulted

Table 1 Specific activity (nmol min⁻¹ mg⁻¹) of homogeneous rat and human glutathione S-transferases

Enzyme	Benzo(α)pyrene-4,5-oxide*		Alkyl epoxide†	Bromosulphophthalein‡
	Exp. 1	Exp. 2		
Rat transferase A	100	88	100	530
B	14	11	0§	6
C	127	99	0§	18
E	51	70	6,100	0§
AA	8	4	0§	4
Human transferase β	25	—	0§	10
δ	33	29	0§	1

*Incubation was for 2 min at 37 °C in 0.4 ml of 10 mM Tris-chloride, pH 7.5, 50 μM ³H-benzo(α)pyrene 4,5-oxide in 15 μl of methanol and 0.5 mM GSH. After precipitation of protein with 0.5 ml acetone and centrifugation, the solution was subjected to thin-layer silica gel chromatography with *n*-butanol-*n*-propanol-2 M ammonium hydroxide (2:1:1). Radioactivity in the R_f 0.06 component was measured⁷. Two series of experiments were conducted with different amounts of the same enzyme. The values presented have been corrected for the small amount of conjugate, about 1 pmol min⁻¹ mg⁻¹, produced in the absence of enzyme.

†Specific activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane as described before¹⁰.

‡Determined spectrophotometrically as described before¹².

§A value of zero implies that nanomolar concentrations of enzyme did not produce conjugate in an amount greater than that of a control in the absence of enzyme.

in the formation of the GSH conjugate (Table 1). Two other substrates of the enzymes are included for comparison: an alkyl epoxide, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and an aryl halide of pharmacological significance, bromosulphophthalein. Both compounds form the appropriate thioethers of GSH. Each of the purified enzymes was active in the conjugation of the polycyclic hydrocarbon epoxide with glutathione although relative activity varied considerably (Table 1). We stress that available information on the rat and human transferases (Kamisaka *et al.*, unpublished) suggests that these enzymes represent a large portion, 10% and 2%, respectively, of the total protein extractable from liver. Furthermore, one of the enzymes, transferase B¹⁴, is identical to ligandin¹⁵, a protein known to form a covalent derivative *in vivo* with 4-dimethylaminoazobenzene¹⁶. Knowledge of the role of the glutathione S-transferases should clarify our understanding of the routes of carcinogen activation and detoxification.

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- ¹ Particulate polycyclic organic matter committee on biological effects of atmospheric pollutants (National Academy of Sciences, Washington, DC, 1972).
- ² Gelboin, H. V., *Cancer Res.*, **10**, 1–81 (1967).
- ³ Wattenberg, L. W., *Cancer Res.*, **26**, 1520–1526 (1966).
- ⁴ Gelboin, H. V., Kinoshita, N. and Wiebel, F. J., *Fedn Proc.*, **31**, 1298–1309 (1972).
- ⁵ Sims, P., and Grover, P. L., *Adv. Cancer Res.*, **20**, 165–274 (1974).
- ⁶ Leutz, J., and Gelboin, H. V., *Archs Biochem. Biophys.* (in the press).
- ⁷ Nemoto, N., and Gelboin, H. V., *Archs Biochem. Biophys.* (in the press).
- ⁸ Boyland, E., and Chasseaud, L. F., *Adv. Enzymol.*, **32**, 173–219 (1969).
- ⁹ Wood, J. L., in *Metabolic conjugation and metabolic hydrolysis* (edit. by Fishman, W. H.), **2**, 261–299 (Academic New York, 1970).
- ¹⁰ Fjellstedt, T. A., Allen, R. H., Duncan, B. K., and Jakoby, W. B., *J. biol. Chem.*, **248**, 3702–3707 (1973).
- ¹¹ Hayakawa, T., LeMahieu, R. A., and Udenfriend, S., *Archs Biochem. Biophys.*, **162**, 223–230 (1974).
- ¹² Habig, W. H., Pabst, M. J., and Jakoby, W. B., *J. biol. Chem.*, **249**, 7130–7139 (1974).
- ¹³ Pabst, M. J., Habig, W. H., and Jakoby, W. B., *J. biol. Chem.*, **249**, 7140–7150 (1974).
- ¹⁴ Habig, W. H., *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3879–3882 (1974).
- ¹⁵ Litwack, G., Ketterer, B., and Arias, I. M., *Nature*, **234**, 466–467 (1971).
- ¹⁶ Ketterer, B., Ross-Mansell, P., and Whitehead, J. K., *Biochem. J.*, **103**, 316–324 (1967).

reviews

OVER the past decade there has appeared a number of books devoted to the subject of exobiology in general, and extra-terrestrial technological communities in particular. The subject is exquisitely fascinating, provocative, and distinguished by a current lack of concrete observational data. These ingredients form a fertile situation for all sorts of unsound speculation so that it is particularly important to have available books on the subject written by competent scientists with established reputations.

One such individual is Ronald Bracewell, of Stanford University, who was among the first contributors to the emergence of scientific consideration of extraterrestrial 'intelligent' communities and the problems and possibilities of radio communication with them. In what Bracewell clearly regards as the pioneering work on the subject, Giuseppe Cocconi and Phillip Morrison suggested in 1959 that radio signals from a technical community in outer space might be directed at us at 1,420 MHz, a natural choice of frequency corresponding to the emission from hydrogen.

Following this proposal, Bracewell detailed three distinct cases for consideration: abundant life, with the nearest community within 30 light years; sparse life within say 30–300 light years; and rare life (beyond 300 light years). He pointed out an important connection between the distance of the nearest community, and the average lifetime of the community. If this lifetime is to be measured in many millions of years, then life in the galaxy may well be abundant.

These arguments, together with some technical considerations about radio

Intelligent life in space

P. C. W. Davies

The Galactic Club: Intelligent Life in Outer Space. By Ronald N. Bracewell. Pp. 141. (Freeman: San Francisco and Reading, 1975.) Cloth £3.70; Paper £1.90.

telescopes and search patterns, are presented in a short volume called *The Galactic Club*, a term which refers to the expected network of information exchange between galactic communities. This is a well written and interesting book, with a rather unusual presentation. Many full page paintings and lithographs of a distinctive style punctuate the text, though their connection with the subject matter is occasionally obscure.

The book encompasses a rather odd conjunction of topics. After a brief introduction to the astronomical, chemical and biological basis of exobiology, Bracewell resurrects the Velikovsky affair recalling that author's fantastic speculations about Solar System cataclysms. Returning to the main theme, some discussion is presented on the exact procedure whereby mankind might intercept interstellar messages, the nature of the message and the response. A chapter is devoted to the ambitious and awesome scheme known as Project Cyclops which proposes a vast array of radio telescopes all busily skimming the sky for messages.

Another chapter is devoted to the remarkable idea of interstellar messenger

probes. Based on the assumption that near-luminal space probes are unlikely to be a practicable possibility at any level of technological attainment, it is suggested that, rather than indulge in interstellar travel themselves, intelligent space people would send out small probes at modest speed, to lie in wait around likely looking stars in the hope of eventually contacting a nascent technical community. The strategy adopted by such an interloper would be to echo domestic radio messages, thereby attracting the attention of an assured audience. The idea is bound to provoke the thought: is there such a probe in the region of the Earth even now?

This type of discussion leads quite naturally to the question of whether the Earth has been (or still is) the recipient of extraterrestrial visitation. In this context, some of the highly popular and evidently fanciful claims of Erich Von Daniken are briefly mentioned, with the conclusion that they satisfy a deep sociological need and "represent a substitution of faith for reason".

Further spectacles are provided by an outline of Gerard O'Neill's amazing studies of spinning space cylinders. These monster stations, envisaged as many miles across, are one idea of a future space colony.

The Galactic Club will have a considerable appeal to popular readership, providing as it does an intellectual journey into a strange and wonderful world of cosmic speculation.

To the scientific community it presents an interesting background to astronomy and space science and, at a more fundamental level, serves to remind us all of a wider perspective of human society.

DISPERSION relations have been a part of the armoury of high energy physics for nearly 20 years now. It may seem surprising that not very many monographs have been exclusively devoted to this subject over the years. I believe that even a casual reading of this latest offering from Drs Queen and Violini will provide a clue as to why that is; on its own the subject can be rather uninspiring, and the authors have not succeeded in showing otherwise. Indeed, the text is written in such a dry, sparse manner with little or no indication of the excitement inherent in fundamental particle physics that I would never dare recommend it as a first introduction to my graduate students. Having said this, I must say that the book is well organ-

Dry dispersion

P. G. Williams

Dispersion Theory in High-Energy Physics. By N. M. Queen and G. Violini. Pp. xi+202. (Macmillan: London and Basingstoke, January 1975.) £12.00.

ised, very carefully written and contains a useful combination of material not previously available in book form.

After a short introduction to scattering formalism the authors obtain dispersion relations from causality, studiously avoiding any complications. Then follow two chapters, one on the Mandelstam representation, the other a rather nice treatment of high energy bounds. Thereafter, we find a six-

chapter catalogue of various types of dispersion relations (excluding partial wave dispersion relations by explicit choice) and their applications. This is relieved by a chapter on Regge theory. At least I had hoped it would be until I read it and found the subject dried out to its very bones by its rather formal treatment. The final chapter, on the analytic continuation techniques of Cutkosky and Deo, is rather useful.

I can't say I welcome this new addition to the literature: the essential parts of the book are better suited to a review article; sections added by way of introduction for students are formal and not at all well physically motivated. And it is all at the astronomic price of £12.00 for a mere 200 pages.

Shedding light

The Theory of Polarization Phenomena. By B. A. Robson. Pp. viii+119. (Clarendon: Oxford; Oxford University Press: London, January 1975.) £5.50.

THIS book is one in the series of monographs *Oxford Studies in Physics*. It comprises just over 100 pages and contains only 40 references in its bibliography. It is therefore very selective in its material and is likely to be narrow in its range of utility. On the other hand, although a vast number of experimental measurements have been obtained there is a paucity of coherent theoretical treatments, so any light (polarised or otherwise) that can be shed on the subject is welcome.

The content of the book is aimed at the gap between undergraduate treatments and research review articles, and it will undoubtedly have most value for new researchers in the field. Starting from a classical basis, the book develops both the Jones and Mueller calculi. The comparison between the two is most valuable. The generality of the Mueller calculus which uses observable quantities and does not rely on the electromagnetic theory of light makes for a powerful and pragmatic description but the Jones calculus has an appealing simplicity for application to coherent beams and polarised light.

Assuming a background knowledge of quantum theory and matrix mechanics, the author develops the quantum theory of polarisation and then gives separate sections on spin $\frac{1}{2}$ and spin 1 particles in the non-relativistic limit. These treatments will be of increasing value as polarised beams of deuterons and other ions become available. The generalisation to the cases of arbitrary spin and polarised targets is of intrinsic

value in view of the advent of superconducting magnets. Further sections include the emission and absorption of electromagnetic radiation and an indication of how to include relativistic effects.

Although my overall impression is of a neat and concise presentation, the complete absence of any experimental considerations will perhaps limit the ultimate value of this book. The few diagrams are of vectorial representations or of energy level schemes which leave much of the physical, three-dimensional aspects of the subject to the imagination.

What is now needed is another monograph to explain the physical meaning of this erudite formalism.

Richard J. Griffiths

Animal ultrasound

Ultrasonic Communication by Animals. By Gillian Sales and David Pye. Pp. x+281. (Chapman and Hall: London, September 1974.) £4.95.

THERE are good physical reasons why the wavelengths, and inversely the frequencies, of sounds generated and received by animals should be related to the size of those animals. Thus, it is no surprise that some of the smaller mammals use ultrasound in communication in the same ways that larger animals use sounds audible to man. But in addition to that a new field is opened up because those shorter wavelengths allow a much stronger interaction with the environment. Some of the technical feats involved in the use of ultrasound are truly remarkable: in addition to the echo-locating behaviour of bats there are a whole variety of evolutionary adaptations such as the ultrasonically silent aerodynamics of the owl,

the ingenious sound generating mechanisms of insects such as grasshoppers, crickets and some species of moth, and the recent and varied developments in moths to detect and avoid bats, newcomers on the evolutionary scene.

Bats themselves produce a wide variety of signals depending on species and circumstances. Undoubtedly, the details of these will intrigue the technically minded reader, and those less technically minded will find the introductory sections of the book useful in explaining ultrasound, its interactions with obstacles, and its detection. It is only fair to point out, however, that only half of the material will interest such a reader. The remainder is clearly intended for the biologist and behavioural scientist.

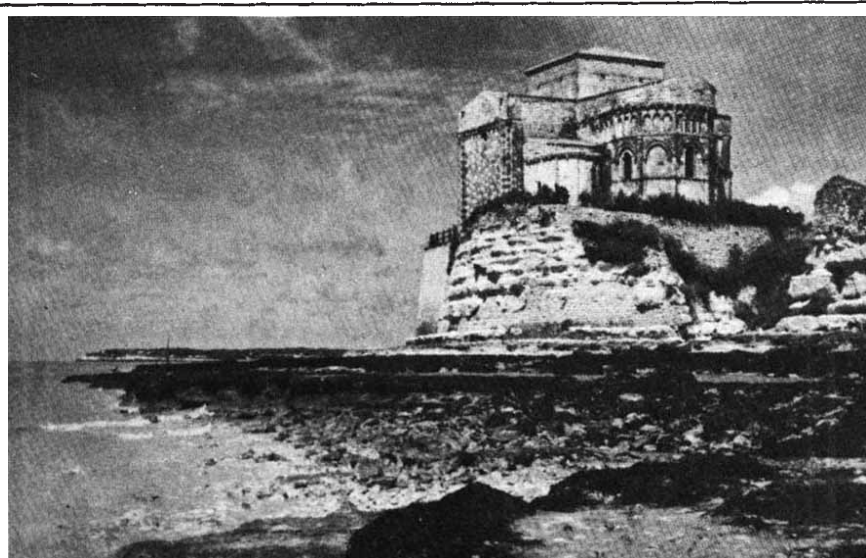
It might be argued that echo-location or navigation by sound is not communication and that the attempt to combine this with true communication leads to difficulties. It seems very arbitrary, for example, to limit discussion of communication between animals to a consideration of ultrasonic frequencies. Presumably, an integrated consideration of *all* sounds audible to the animals concerned, and comparison with other species not using ultrasound, would give much greater insight into the subject.

Such limitations are less obvious on the echo-location side as it is necessary to use the short wavelengths of ultrasound to detect small objects. In any case, the authors discuss examples of navigation using audible frequencies by the oil bird and cave swiftlet. One of the problems which faces the authors is the large number of species which have been researched, each with subtle variations in signals and behaviour, so that a comprehensive picture inevitably becomes repetitive.

The book naturally emphasises the authors' interests: bats, rodents, crickets and related insects. They devote much less attention to marine animals which indulge in reasonably sophisticated communication and rival in some respects the navigational abilities of bats. An area which stands out for future investigation is passive echo-location using ambient sounds which could in principle be much more effective at ultrasonic frequencies for small animals than for the blind man.

Generally, the photographic and line illustrations are adequate, but the oscillographs and sonograms have reproduced poorly and are not helped by the inartistic patterns of black masking. The book is well referenced and contains a wealth of information on animal signals that have only recently become accessible to man. Perhaps other readers like me will be tempted to construct bat detectors of their own.

J. P. Wilson



An 11th century church at Talmot in France. From *The Conservation of Cities*. Published as a contribution to European Architectural Heritage Year. Pp. 186. (Croom Helm: London; UNESCO: Paris, 1975.) £4.95.